FUNCTIONAL STUDIES OF LIGNIN BIOSYNTHESIS GENES AND PUTATIVE FLOWERING GENE IN *MISCANTHUS* X *GIGANTEUS* AND STUDIES ON INDOLYL GLUCOSINOLATE BIOSYNTHESIS AND TRANSLOCATION IN *BRASSICA OLERACEA*

BY

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DISSERTATION

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ABSTRACT

The first topic area of this thesis involved studies on the accumulation and translocation of glucosinolates (GSs), bioactive secondary plant compounds, in broccoli plants. Changes in GS accumulation and gene expression levels in response to exogeneous methyl jasmonate (MeJA) treatment were analyzed in different tissue types at different developmental stages of broccoli. Greater accumulation of GSs with MeJA treatment was observed in apical leaves of broccoli seedlings and florets of plants at harvest maturity. Increases in indolyl GS in apical leaves of seedlings and florets were coupled with the up-regulation of indolyl GS biosynthesis genes. The accumulation of indolyl GSs appears to be modulated by MeJA treatment in an organ-specific manner for optimal distribution of defense substances in the plant. Metabolic profiling of hydrophilic metabolites using GC-MS demonstrated increased accumulation of various phenolics, ascorbates and amino acids in broccoli tissues after MeJA treatment. Distinct changes in carbohydrate levels observed between different tissues (vegetative leaves and floret tissues) of broccoli plants after treatment suggest that carbon metabolism is differentially modulated by MeJA treatment in different tissue types depending on sink-source relationships. Reduced levels of hexose sugars and tricarboxylic acid intermediates after MeJA treatment may reflect the increased requirement for carbon and energy needed to drive secondary product biosynthesis to accumulate metabolites for defense against insects and other herbivores.

Substantial increases of indolyl and aromatic GSs after exogenous treatment with MeJA in stem and petioles of seedlings and the existence of intact indolyl-GS forms in phloem exudates suggest enhanced *de novo* synthesis in combination with active transport. Indoly GSs share structural similarities with the auxin, IAA, and may interact with components of the auxin transport system for intra- and extra-cellular transport or translocation. Application of the auxin

efflux inhibitor, 1-naphthylphthalamic acid (NPA) reduced MeJA-mediated accumulation of indolyl GSs in broccoli florets and seedling tissues. NPA did not inhibit expression of indolyl GS biosynthesis genes shown to be upregulated by MeJA treatment or the accumulation of tryptophan, the amino acid precursor of indolyl GSs. Exogenous application of benzyl GS to *Arabidopsis* roots induced ectopic expression of the PIN1 protein associated with the auxin transport system similar to treatment with NPA, again suggesting GS interaction with the auxin efflux carrier system. The inhibitory effect of NPA on MeJA-mediated accumulation of GS may be due to competitive binding of NPA to auxin efflux carrier components and that GS transport is mediated by the auxin transport system. The inhibitory effect of NPA on indolyl and aromatic GS accumulation and the bioactivity of exogenous treatment of these GS compounds in PIN1 localization, *Arabidopsis* root growth, and gravitrophic response suggest that indolyl and aromatic GSs may be antagonistic to IAA transport and biosynthesis. Indolyl and aromatic GSs can also be potentially converted into IAA by hydrolysis. This intrinsic feature of GSs may be the part of a sophisticated regulatory process where the metabolic pathways in the plant shift from active growth to a reversible defense posture in response to biotic or abiotic stress. It seems likely that indolyl and aromatic GSs are important compounds that provide connections between jasmonate and auxin signaling. Further studies are required to reveal the regulatory mechanism for crosstalk between the two hormones.

The third part of this research was to investigate effect of selenium fertilization and MeJA treatment on accumulation of GSs in broccoli florets. Increasing dietary intake of the element selenium (Se) has been shown to reduce the risk of cancer. Simultaneous enhancement of both Se and GS concentrations in broccoli floret tissue were conducted through the combined treatment of MeJA with Se fertilization. A low level of Se fertilization (concentration) with

MeJA treatment displayed no significant changes in total aliphatic GS concentrations with 90% and 50% increases in indolyl and total GSs concentrations, respectively. This result suggests that Se- and GS-enriched broccoli with improved health-promoting properties can be generated by this combined treatment.

The second topic of this thesis was conducted to provide basic information required to improve biomass quality and productivity and develop tools for gene transformation in *Miscanthus* x *giganteus*. The perennial rhizomatous grass, *Miscanthus* x *giganteus* is an ideal biomass crop due to its rapid vegetative growth and high biomass yield potential. As a naturally occurring sterile hybrid, *M.* x *giganteus* must be propagated vegetatively by mechanicalling divided rhizomes or from micropropagated plantlets. The effect of callus type, age and culture methods on regeneration competence was studied to improve regeneration efficiency and shorten the period of tissue culture in *M.* x *giganteus* propagation.

Seven lignin biosynthesis genes and one putative flowering gene were isolated from *M.* x *giganteus* by PCR reactions using maize othologous sequences. Southern hybridization and nuclear DNA content analysis indicated that the genes isolated from *M.* x *giganteus* exist in the genome of other *Miscanthus* species as multiple copies. Analysis of lignin content and histological staining of lignin deposition indicated that higher lignin content is found in mature stem node tissues compared to young leaves and apical stem nodal tissues. Cell wall lignification is associated with increasing tissue maturity in *Miscanthus* species. RNAi and antisense constructs harboring sequences of these genes were developed to generate *Miscanthus* transgenic plants with suppressed of lignin biosynthesis and delayed flowering.

To my wife, Sang A Park

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TABLE OF CONTENTS

STUDIES ON INDOLYL GLUCOSINOLATE BIOSYNTHESIS AND TRANSLOCATION IN *BRASSICA OLERACEA*

CHAPTER 1

Literature Review

1.1. Glucosinolates

Glucosinolates (GSs), nitrogen- and sulfur-containing phytochemicals, are biologically active secondary metabolites primarily found in the family Brassicaeae, although other GS-containing plant genera occur within the families Capparaceae and Caricaceae (Fahey et al., 2001). GSs have a common structure comprising a sulfonated oxime and a β-thioglucose residue, and a variable side chain of modified amino acids which is responsible for their chemical diversity (Fahey et al., 2001; Rodman et al., 1998; Wittstock and Halkier, 2002). GSs are divided into three classes based on the amino acid precursors from which they originate (Fenwick et al., 1983). Aliphatic GSs are derived from methionine, while indolyl and phenyl GSs are derived from tryptophan and phenylalanine, respectively. Seventeen different GSs have been identified among *Brassica* species (Table 1.1). These GSs are hydrolized by the plant endogeneous enzyme myrosinase into nitrile, isothiocyanate, or thiocyanate forms that are responsible for their various biological functions (Wittstock and Halkier, 2002).

1.2. GS biosynthesis

The GS biosynthetic pathway has been well studied in *Arabidopsis*, and many of the associated genes have been isolated and characterized through *in vitro* biochemical assays and mutant studies (Figure 1.1) (Hull et al., 2000; Mikkelsen et al., 2000; Wittstock and Halkier, 2000; Hemm et al., 2003; Celenza et al., 2004; Mikkelsen et al., 2004; Tantikanjana et al., 2004). The pathway is divided to primary three parts: (i) side chain elongation of amino acids, (ii) formation of the core structure, and (iii) secondary modifications of the side chain generating chemical diversity. A side chain amino acid precursor undergoes a series of chain elongations

prior to entering the pathway. Two genes, *methylthioalkylmalate synthase 1* (*MAM1*) and *MAM2*, have been identified in *Arabidopsis*, where *MAM1* regulates the first two methionine elongation cycles, while *MAM2* results in only one round of elongation (Textor et al., 2004; Kroymann et al., 2003). The second step is the GS core structure formation from the precursor amino acids by reaction with various cytochrome P450 enzymes (CYP) (Halkier, 1999). CYP79 catalyze the conversion of amino acid to aldoximes. *CYP79F1* and *CYP79F2* genes are responsible for aldoxime production leading to aliphatic GSs derived from chain-elongated methionone derivatives, whereas *CYP79B2* and *CYP79B3* have distinct functions for indolyl GS biosynthesis derived from tryptophan. The conversion of aldoximes to thiohydroximic acids is catalyzed by the aldoxime-metabolizing enzyme, CYP83. Aliphatic aldoximes are primary metabolized by CYP83A1, whereas indolyl and aromatic aldoximes derived from tryptophan, phenylalanine, and tyrosine are metabolized by both CYP83A1 and CYP83B1 (Reviewed in Halkier and Gershenzon, 2006). The initially formed parent GS is subjected to a wide range of modification including hydroxylation, methylation, and oxidation, giving rise to the structural diversity of these compounds found in plant tissues. In *Arabidopsis*, two genes encoding 2-oxoacid-dependent dioxygenases were functionally characterized as the gene controlling oxidation of the side chain (Kliebenstein et al., 2001). The *alkenyl/hydroxypropyl loci 2* (*AOP2*) gene product was found to convert methylsulfinylalkyl to alkenyl glucosinolates when heterologously expressed in *E. coli* and showed distinct expression only in ecotypes accumulating alkenyl GSs. The *AOP3* gene product, expressed only in ecotypes accumulating hydroxyalkyl GSs, converted a methylsulfinylalkyl to a hydroxyalkyl GS (Kliebenstein et al., 2001).

1.2.1. Indolyl GS biosynthesis

Indolyl GS is an important class of tryptophan (Trp) secondary metabolites with a biosynthetic pathway that branches with the auxin biosynthetic pathway (Figure 1. 1) (Bak and Feyereisen, 2001). In the biosynthetic pathway of indolyl GS, CYP79B2 catalyzes the conversion of tryptophan to indole-3-acetaldoxime, with CYP83A1 and CYP83B1 metabolizing the phenylalanine- and tyrosine-derived aldoximes. Indolyl GS biosynthesis is induced by the methyl jasmonate (MeJA) and the salicylic acid (SA) signal transduction pathways (Mikkelsen et al., 2003). MeJA treatment of *Arabidopsis* increased the amount of indolyl GSs by 3- to 4-fold with highly induced expression of the corresponding Trp-metabolizing genes *CYP79B2* and *CYP79B3*, whereas few changes were seen for the levels of aliphatic GSs. *CYP79B2* showed wound-inducible expression in leaves, stem, flowers, and roots of *Arabidopsis* (Mikkelsen et al., 2000). Strong expression of *CYP79B2* was associated with accumulation of indolyl GSs in *Arabidopsis* root tissue (Brown et al., 2003). Over-expression of *CYP79B2* resulted in a significant increase in indolyl GS concentrations in transgenic *Arabidopsis* (Mikkelsen et al., 2000). Recently, another *P450* gene, *CYP81F2*, was identified as the *Indole Glucosinolate Modifier1* (*IGM1*) gene through QTL fine-mapping and microarray-based transcript profiling in *Arabidopsis*. CYP81F2 catalyzes the conversion of indolyl-3-methyl to 4-hydroxyindolyl-3-methyl GS which was verified by analysis of *Arabidopsis* T-DNA knockout mutants (Pfalz et al., 2009).

Several transcription factors involved in indolyl GS biosynthesis have been isolated and identified in *Arabidopsis* (Skirycz et al., 2006; Levy et al., 2005; Celenza et al., 2005). *IQD1* encodes a nuclear-localized calmodulin-binding protein has been reported to upregulate genes related with the indolyl GS biosynthesis. Gain- and loss-of-function *IQD1* gene mutants resulted

in increases and decreases in GS levels in *Arabidopsis*, respectively (Levy et al., 2005). AtDof1.1, a member of the DNA-binding-with-one-finger transcription factor family, has been shown to upregulate the *CYP83B1* gene, leading to the increase in indolyl GS levels in *Arabidopsis* (Skirycz et al., 2006). *AtDof1.1* expression was activated in response to wounding and herbivore attack and by treatment with the MeJA. ATR1, a Myb transcription factor, activated the expression of *CYP79B2*, *CYP79B3* and *CYP83B1*. Overexpression of *ATR1* elevated IAA and indolyl GS levels in *Arabidopsis* (Celenza et al., 2005). *ATR1* has been proposed to control homeostasis between IAA and indolyl glucosinolate biosynthesis.

1.3. GSs and their hydrolysis products

Most GSs are chemically and thermally stable. Enzymatic hydrolysis involves myrosinase, a glycosylated thioglucosidase that cleaves the glucose unit from the GS to form an unstable intermediate, which can undergo rearrangement into several different biological active compounds such as isothiocynates, nitriles or thiocyanates (Figure 1.2) (Fenwick et al., 1983). In plant tissues, myrosinase is present in idioblastic myrosin cells and physically separated from its substrates. Compartmentalization of the GS-myrosinase system has been shown in *Arabidopsis* by the identification of sulfur-rich cells (S-cells) between the phloem and the endodermis of the flower stalk, which presumably contain high concentrations of GSs (Koroleva et al., 2000), and by the localization of myrosinase in neighbouring cells (Andreasson et al., 2001). When the plant tissue is disrupted, myrosinase and substrate (GSs) come into contact, and consequently results in GS hydrolysis. The chemical structure of hydrolysis products depends on the structure of the GS side chain and reaction conditions such as pH, concentration of Fe^{2+} and presence of epithiospecifier protein (ESP) (reviewed in Holst and Williamson, 2004). At neutral pH, the major hydrolysis products are ithothiocyanates while nitriles are the dominant products at acidic

pH. GS containing terminally unsaturated side chains such as a terminal double bond produces epithionitriles when degraded in the presence of the ESP and Fe^{2+} ions. In the absence of ESP, the addition of Fe^{2+} ions also promotes nitrile formation (Matusheski and Jeffery, 2001; Foo et al., 2000).

The ESP proteins (non-catalytic cofactors of myrosinase) have been demonstrated to divert the outcome of the myrosinase-catalyzed hydrolysis towards nitriles or epithionitriles without having hydrolytic activity on GSs themselves (Burow et al., 2008; Lambrix et al., 2001; Matusheski et al., 2006). The *epithiospecifier modifier 1* (*ESM1*) gene encodes a protein shown to inhibit function of ESP, leading to increased isothiocyanate production from GS hydrolysis (Zhang et al., 2006; Burrow et al., 2008). Aglycones containing β–hydroxylated side chains such as 2-hydroxy-3-butenyl (progoitrin) spontaneously cyclize to oxazolidine-2-thiones, a class of substances known to cause goiter. Thiocyanates are formed from only three GSs: benzyl-, allyl-, and 4-methylsulfinylbutyl GS.

Indolyl GSs also form unstable aglycones which can form either the corresponding nitriles or isothiocyanates (Figure 1.3) (Chevolleau et al., 1997; Agerbirk et al., 1998). Formation of other immediate products such as organic thiocyanates and epithionitriles from indolyl glucosinolates has not been shown. The fragmentation and rearrangement of indolyl alycones into isothiocyanates or nitriles are influenced by the same factors, notably pH, ESP, and Fe^{2+} ions as with the aliphatics (Burow et al., 2007). Indolyl GSs can also form indolyl-3-acetonitrile. This nitrile has auxin activity, and can also be converted to indole-3-acetic acid by nitrilase. All isothiocyanates produced from indolyl GSs are unstable due to a replacement of the functional group with various nucleophiles. This reactivity of indolyl-3-methyl isothiocyanate causes the formation of a large variety of indolyl-containing downstream products such as ascorbigen and

indole-3-carbinol. These two major immediate products, ascorbigen and indole-3-carbinol, are formed by reaction of indolyl-3-methyl isothiocyanate with ascorbic acid and water, respectively (Reviewed in Agerbirk et al., 2009).

1.4. Biological properties of GS hydrolysis products

Various GS hydrolysis products generated through the GS-myrosinase system have different biological activities, such as plant defense functions against generalist herbivores and pathogens, and human anti-carcinogenic bioactivity (Kirkegaard and Sarwar, 1998; van Poppel et al., 1999; Mithen et al., 2000; Noret et al., 2005). The roles of the GS hydrolysis products have received considerable attention due to their potentials as human cancer-prevention agents, crop protection compounds, and biofumigants in agriculture (Halkier and Gershenzon, 2006)

1.4.1. GSs and human health

Epidemiological studies have demonstrated that increased consumption of cruciferous vegetables in human diets is associated with a reduced risk of a number of cancers (Verhoeven et al., 1996). Studies in animals also have shown that, when consumed in normal quantities, GSs inhibit the neoplastic effects of various carcinogens at a number of organ sites. *In vitro* studies conducted to elucidate the mechanism for this effect have indicated that isothiocyanates enhance the activity of detoxification enzymes and enzymes capable of repairing DNA damage (Talalay and Fahey, 2001). Isothiocyanates can also inhibit cancer cell division and growth (Gamet-Payrastre et al., 2000), enhance apoptosis of pre-cancerous cells containing damaged DNA (Conaway et al., 2002), function as direct or indirect antioxidants decreasing oxidative stress (Zhu et al., 2000; Zhu and Loft, 2001), modulate cell signaling (Xu and Thornalley, 2001), and act as antibiotics in *Helicobacter* infections (Fahey et al., 2002).

GS hydrolysis products are classified as mono or bi-functional inducers of phase I and II detoxification enzymes depending on whether one or both phase enzymes are upregulated by each GS hydrolysis product (Talalay and Zhang, 1996). The phase I enzymes, mostly P450s, functionalize xenobiotics by oxidation or reduction reactions. Phase II enzymes, including glutathione-S-transferase (GST) and quinone reductase (QR), act to conjugate functionalized products with endogenous ligands or destroy reactive factors (Williams, 1971). Carcinogens modified by these detoxification enzymes are less reactive and more rapidly excreted (Wattenberg, 1985). Isothiocyanate-mediated changes in detoxification enzyme systems have been hypothesized to reduce cancer risk by inhibiting activation of pro-carcinogens and inducing excretion of carcinogens (Talalay and Fahey, 2001).

The most intensively investigated isothiocyanates are sulforaphane, phenethyl isothiocyanate, allyl isothiocyanate and indole-3-carbinol (Hecht, 1999). Sulforaphane, the isothiocyanate derived from 4-methylsulphinylbutyl GS (glucoraphanin), is highly potent at upregulating detoxification enzymes in cell culture. Sulforaphane may also interfere with cancer promotion and progression via apoptosis (Zhang and Talalay, 1994; Gamet-Payrastre et al., 2000). Phenylethyl isothiocyanate (PEITC) is derived from 2-phenylethyl glucosinolate (gluconasturtiin). Chinese cabbage, radish, and watercress are rich sources of PEITC (Chung et al., 1992). PEITC has been shown to increase QR and GST activity in rats (Guo et al., 1992; Staack et al., 1998). Allyl isothiocyanate (AITC), an isothiocyanate derived from 2-propenyl GS (sinigrin), significantly inhibits the proliferation of cultured androgen-independent and dependent human prostate cancer cells in a dose-dependent manner (Xiao et al., 2003). Reduced proliferation of androgen-independent and dependent human prostate cancer cells in the presence of AITC correlates with the concentration of cells in the G_2/M phase and the induction of

apoptosis. Indole-3-carbinol, the hydrolysis product of the 3-indolylmethyl GS (glucobrassicin), has proven successful in National Cancer Institute clinical trials against breast cancer (Telang et al., 1997) and respiratory papilloma (Rosen et al., 1998). Indole-3-carbinol has been reported to act as a chemoprotector by bifunctional upregulation of detoxification enzymes in humans (Wortelboer et al., 1992). Indole-3-carbinol was found to induce apoptosis and cell cycle arrest at the G_1 cell cycle phase in human prostate cancer cell lines (Nachshon-Kedmi et al., 2003). The hydrolysis product of *N*-methoxyindolyl-3-methyl GS, *N*-methoxyindole-3-carbinol also demonstrated similar anticarcinogenic activites (reviewed in Aggarwal and Ichikawa, 2005). *N*-methoxyindole-3-carbinol inhibited the proliferation of human colon cancer cell lines through the delay of the G_1-S phase transition of cancer cell lines (Neave et al., 2005). *N*-methoxyindole-3-carbinol was observed to be a more efficient inducer of the CYP1A1 detoxification enzyme in cultured cell lines than indole-3-carbinol (Stephensen et al., 2000).

1.4.2. Selenium fertilization

The element selenium (Se) is an essential micronutrient for animals and humans (Rayman, 2004; White and Broadley, 2005). It has been reported that Se deficiency in humans is associated with cardiovascular disorders, hypothyrodism, a weakened immune system, male infertility, and increased incidence of various cancers (Gupta and Gupta, 2002; Rayman, 2002; Whanger, 2004; Finley, 2005; reviewed in White et al., 2007). Consumption of crops grown in Se-deficient soils suggested that 15% of the world's population are Se deficient (Rayman, 2002; White and Broadley, 2005). The current recommended dietary allowance (RDA) for human in the USA is 55 μg (0.7 μmol) of Se per day (Rayman, 2004; White and Broadley, 2005). Dietary supplementation of 200 μg Se per day has been demonstrated to reduce cancer risk in humans (Clark et al., 1996). Consumption of Se-enriched broccoli demonstrated the enhanced protection

against colon and mammary cancer in laboratory animals compared to control broccoli alone (Finley et al., 2000; Davis et al., 2002; Finley et al., 2001). Se is also associated with the activity of several important antioxidant enzymes such as the gluthathione peroxidase isozymes and thioredoxin reductase (Brown and Arthur, 2001). When Se is consumed in large quantities, issues related to toxicity arise (Raisbeck, 2000; Vinceti et al., 2001).

Se can not be added to food directly since it is not on the FDA's generally recognized as safe (GRAS) list (Finley et al., 2005). Therefore, it can be enriched naturally through the Se fertilization leading to the accumulation and concentration of Se in crop species. Plant species can be classified into three groups, Se non-accumulators, Se-indicators, and Se-accumulators, depending on their ability to assimilate and accumulate Se (Rosenfeld and Beath, 1964; Brown and Shrift, 1982; White et al., 2004). Plant species which can not tolerate tissue Se concentrations above 10-100 μg Se g^{-1} dry matter are termed as Se non-accumulators (Rosenfeld and Beath, 1964; White et al., 2004). Se-indicator plants can be grown in both seleniferous and nonseleniferous soils, where they can accumulate up to 1,000 μg Se g^{-1} dry matter in their tissues. Se levels in accumulator species can contain up to 15,000 μg Se g^{-1} dry matter when they grow in seleniferous soils (Rosenfeld and Beath, 1964).

Both Se and sulfur are belong to Group VI of the periodic table and have similar chemical properties. Root uptake and transport of Se and sulfur is a competitive process. Fertilization of both Se and sulfur inhibites Se uptake and accumulation in plants, indicating direct competition between Se and sulfur, whereas increasing Se concentration in the soil can increase shoot sulfur concentrations through feedback mechanisms regulated by plant sulfur status (White et al., 2004). Se is assimilated into organic compounds through the sulfur assimilation pathway (Hawkesford, 2005; Hawkesford and De Kok, 2006).

1.4.3. GSs and plant defense

GSs are present constitutively in plant tissues, but are also rapidly induced to higher levels by herbivore attack. Among the various GS types, indolyl GSs are most frequently induced regardless of the type of herbivore involved (Textor and Gershenzon, 2009). Elevated accumulation of indolyl GSs in the damaged tissues caused by herbivore attack suggest they serve a defensive function. Indolyl GS induction by *Myzus persicae* (Kim and Jander, 2007) and *Pieris rapae* feeding (Mewis et al., 2006) was reported in *Arabidopsis*, and in *Brassica oleracea* by *Delia floralis* feeding (Birch et al., 1992).

In general, GS hydrolysis products are biologically antagonistic to a wide range of herbivores including mammals, insects, mollusks, aquatic invertebrates and nematodes through the exhibition of outright toxicity (Borek et al., 1998; Lazzeri et al., 2004; Li et al., 2000), significant growth inhibition (Agrawal and Kurashige, 2003; Burow et al., 2006), or as general deterrents to herbivore feeding (Newman et al., 1992; Noret et al., 2005; Siemens and Mitchell-Olds, 1996). It is clear that the GS hydrolysis products are responsible for these effects rather than the parent GSs. Direct test of hydrolysis products and elimination of hydrolysis reactions by myrosinase inactivation or breeding for low myrosinase activity clearly showed significantly increased toxicity and deterrence, and reduced activity, respectively (Agrawal and Kurashige, 2003; Borek et al., 1997; Li et al., 2000).

GSs are not effective against all herbivores. The same compounds that poison and deter generalist herbivores may function as feeding or oviposition attractants to specialists (Mewis et al., 2002; Miles et al., 2005). The diamondback moth, *Plutella xylostella*, inactivates GSs by an endogeneous sulfatase reaction preventing hydrolysis of GSs by structural modification (Ratzka et al., 2002). The larvae of the cabbage white butterfly, *Pieris rapae*, modified GS hydrolysis

from isothiocynates to nitriles through synthesis of an endogeneous protein, Nitrile specifier protein (NSP) (Wittstock et al., 2004). Nitriles appear to be less toxic to herbivorous insects than isothiocyanates (Burow et al., 2006).

GS hydrolysis products are also considered as defenses against some microorganisms, especially necrotrophs (Li et al., 1999; Mari et al., 2002; Smith and Kirkegaard, 2002). Induction of GSs, especially indolyl GSs, in response to the attack of fungi and bacteria have been reported (Brader et al., 2001; Rostás et al., 2002; Vierheilig et al., 2000). Recently, an atypical PEN2 myrosinase (a type of β-thioglucoside glucohydrolase) which can hydrolyze 4-methoxyindolyl-3-methyl GS for antifungal defense, was characterized in *Arabidopsis* (Bednarek et al., 2009). With their antimicrobial effects, some *Brassica* species have been used as cover crops that following soil incorporation act to naturally suppress pests and disease organisms in the soil (Kirkegaard and Sarwar, 1998).

1.4.4. Plant defense signaling pathways associated with GS metabolism

Endogenous jamonates (JAs) modulate the expression of genes associated with the plant defense system in response to abiotic and biotic stresses. Several stress conditions such as wounding and pathogen and herbivore attack lead to endogenous JA biosynthesis and the induction of JA-mediated gene expression (Pauwels et al., 2008). Indolyl GSs are particularly induced by the JA and salicylic acid (SA) signal transduction pathways. MeJA, a volatile methylester of JA, has proven to induce the production of GS in *Brassica*, especially that of indolyl GS (van Dam et al., 2003; Doughty et al., 1995; Bodnaryk et al., 1994). After MeJA treatment, the amount of indolyl GSs was increased 3- to 4-fold and up to 10-fold in for *N*-methoxy-3-indolylmethyl GS in *Arabidopsis* leaves. The corresponding Trp-metabolizing genes *CYP79B2* and *CYP79B3* were also both highly induced (Mikkelsen et al., 2003). In this report,

aliphatic GSs did not show significantly increased levels after MeJA treatment, although 8-methylthiooctyl GS and 8-methylsulfinyloctyl GS showed slightly increased levels. Wounding treatments also moderately increased the concentration of 3-indolylmethyl GS, but not as dramatically as in MeJA treated leaf tissues. Shoot application of JA to *Brassica oleracea* increased indolyl GS levels 3- to 20-fold in shoots. Total GS levels in the roots were not increased with shoot JA application. Root application of JA also did not increase indolyl GSs levels in the root, whereas it increased aliphatic GSs in the shoot. Plants treated with JA to both organs (shoot and root) had profiles similar to shoot-treated plants (van Dam et al., 2003).

SA is an essential phytohormone that activates the systemic acquired resistance (SAR) response in plants associated with increased pathogen resistance (Gaffney et al., 1993; Delaney et al., 1994; Mauch-Mani and Métraux, 1998). SA treatment altered GS accumulation in *Arabiodpsis* (Kliebenstein et al., 2002). SA subjected leaf tissue showed increased levels of 4-methoxy-3-indolylmethyl GS without any significant change in levels of other GSs, whereas MeJA treatment increased the level of 3-indolylmethyl GS and N-methoxy-3-indolylmethyl GS. The combination treatment of both MeJA and SA counteracted the effect of each treatment alone. This negative interaction between MeJA and SA has been also observed in other experiments (Cipollini et al., 2005; Gupta et al., 2000).

1.5. Broccoli

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is an economically important vegetable of the family Brassicae. Broccoli has been shown to be a good source of several health promoting phytochemical compounds including GSs, carotenoids, tocopherols, and ascorbic acid (Kushad et al., 1999; Kurilich et al., 1999). The health-promoting properties of broccoli has increased the popularity of this vegetable and provided value-added economic benefits for its commercial

production. Over the last two and a half decades, consumption of fresh broccoli has increased from 1.4 pounds consumed per person in 1980 to 5.9 pounds in 2008 (ERS, 2009). In broccoli florets, the primary GSs are 4-methylsulfinylbutyl GS (glucoraphanin), 3-butenyl GS (gluconapin), and 3-indolylmethyl GS (glucobrassicin) (Kushad et al., 1999). Among these GSs, sulforaphane and indole-3-carbinol, the hydrolysis product of 4-methylsulfinylbutyl GS and 3-indolylmethyl GS, respectively, have shown human anti-carcinogenic bioactivity (Zhang and Talalay, 1994; Telang et al., 1997).

Broccoli is a good model system for studying GS metabolism because it has high concentrations of the various GS compounds compared to *Arabidopsis*. In the same taxonomic family, broccoli shares genetic homology with *Arabidopsis* (Lan et al., 2000). Hence, the sequence information and genetic tools available for *Arabidopsis* can be used for comparative gene functional studies.

1.6. GS concentration and composition in *Brassica* species

The composition and content of GSs have been found to vary widely between different species and at different developmental stages and organs within a given species (Porter et al., 1991; Li et al., 1999; Koroleva et al., 2000). Comparative studies of the most widely consumed cruciferous vegetables on GS concentration and composition showed distinct distribution and variability of GS profiles (Carlson et al., 1987). The primary GSs in cabbage, cauliflower and kale are 2-propenyl GS (sinigrin) and 3-indolylmethyl GS (glucobrassicin). 2-propenyl GS, 3-indolylmethyl GS and 2-hydroxy-3-butenyl GS (progoitrin) are predominant GSs in Brussels sprouts. No 4-methylsulfinylbutyl GS (glucoraphanin) has been detected in cabbage, Brussels sprouts and cauliflower, and only a trace amounts found in kale (Carlson et al., 1987; Kushad et al., 1999; Rosa, 1997; Rosa et al., 1996).

Significant differences of GS concentration and composition were noted among various organs and developmental stages of *Arabidopsis* (Petersen et al., 2002; Brown et al., 2003). Germinating seeds had the highest total GS concentration, followed by young leaves, old leaves and roots. After the transition from vegetative to reproductive stage, total GS concentrations decreased in leaves but increased in inflorescences and siliques (Brown et al., 2003). These patterns are similar to those found in other GS-containing species (Fahey et al., 2001). Palmer et al. (1987) and Rosa et al. (1996) also reported a large decrease of GSs from seedling leaves to mature leaves in *B. juncea*, *B. oleracea* and *B. napus*. The senescence of older leaves may cause disruption of cells leading to GS catabolism. Macfarlane Smith and Griffiths (1988) also reported that the concentration of total aliphatic and aromatic GSs were lower in the leaves than the stems of *B. napus* at all harvest dates. Booth et al. (1991) reported that total GSs in *B. napus* declined in the vegetative tissue then increased in floral tissues and seeds at the onset of flowering and seed formation, respectively. The results from Rosa and Heaney (1996) also showed that in Portuguese cabbage, the concentration of total GS and some individual GSs such as 2-propenyl GS and 2-hydroxy-3-butenyl GS were significantly higher in the heads than in the leaves. These results indicate that GS accumulation tends to be concentrated into more valuable plant organs such as the tender sprout or inflorescence. It has been hypothesized that these organ-specific distributions may be associated with optimal distribution of defense substances to protect against insects and other herbivores (Zangerl and Bazzaz, 1993). For instance, the proportionally dominant 2-phenylethyl GS (aromatic GS) in *Brassica napus* root was associated with the susceptibility of the crop to the root lesion nematode (*Pratylenchus neglectus*) (Potter et al., 2000). Differential distribution of GSs in different organs and compositional changes during different developmental stages provides useful information to understand how the plant regulates

accumulation and distribution of GSs. Treatment of exogenous MeJA or mechanical wounding increasing indolyl GS concentrations systemically in various *Brassica* spices (reviewed in Textor and Gershenzon, 2009) also can be used to investigate the mechanism of plant response against biotic stress through the regulation of GS metabolism.

1.7. GS transport

GSs have been suggested to be actively transported in plants. In *Tropaeolum majus*, benzyl GS primarily synthesized in leaves accumulated in developing seeds, suggesting translocation (Lykkesfeldt and Moller, 1993). GS profiling data of seed and leaf tissue of *B. napus* also showed that the profile of the aliphatic GSs in the seed was identical to the profile in the leaves of the maternal plant (Magrath and Mithen, 1993). Comparison of the GS profiles of *Arabidopsis* leaves, roots and stems from mature plants with those of green siliques and mature seeds suggested that a majority of the seed GSs were synthesized de novo in the silique (Petersen et al., 2002). The vegetative tissues and stem tissue contained predominantly GSs with secondary modifications (e.g. methylsulphinylalkyl and methoxylated indolyl GSs), while siliques and mature seeds contained predominantly high amounts of parent GSs (e.g. methythioalky GSs and 3-indolylmethyl GS). These results suggest that the fully formed GSs are transferred from maternal tissue into developing seeds.

It has been shown that both intact GSs and desulfo-GSs possess the physico-chemical properties allowing for phloem-mediated transport (Brudenell et al., 1999). In developing seedlings of *Brassica napus*, radiolabelled desulfo-GSs fed to silique walls and seedlings, these GS precursors were taken up and converted into GSs, suggesting that desulfo-GSs may be the transported form (Thangstad et al., 2001). However, the presence of intact GSs in the phloem has recently been demonstrated in *Arabidopsis*, indicating intact GSs are transported forms (Chen et

al., 2001). Long-distance transport of GSs was investigated to determine if transport follows the principle of assimilate translocation by mass flow from source to sink. To test this hypothesis, radiolabelled [^{14}C] tyrosine was fed to a rosette leaf of young 35S::CYP79A1 *Arabidopsis* transgenic plants, and then the formation of radiolabelled *p*-hydroxybenzyl glucosinolate was detected. The radiolabelled GS was exported from the leaves to the other rosette leaves, roots, stem, cauline leaves, flower buds and siliques, possibly via transport in the phloem (Chen et al., 2001). Uptake of GSs was observed in *B. napus* embryos and leaf protoplasts for both 2-propenyl GS (sinigrin) and benzyl GS, implying existence of specific GS transporter, which does not differentiate by structure of the side chain (Gijzen et al., 1989; Iqbal and Möllers, 2003; Chen and Halkier, 2000). Although these studies support active transport, regulatory factors and transporter proteins associated with systemic allocation and translocation of GSs via phloem are poorly understood. Recently, the transporter protein for aliphatic GS was identified in *Arabidopsis* (Gigolashvili et al., 2009). This protein, BAT5, acts as a plastidic transporter required for import and export of side chain elongation elements such as 2-keto acids and chain-elongated 2-keto acids. Although the BAT5 is essential component of the aliphatic GS biosynthetic pathway, its function is limited for transport of only aliphatic side chains.

1.7.1. Potential interaction of GSs with auxin transport components

Indole-3-acetic acid (IAA) is the most abundant naturally occurring plant auxin. IAA enters the cytoplasm from the cells wall by diffusion of the protonated form of IAA across the cell membrane and also via membrane-bound influx carriers (AUX/LAX), proton symporters that facilitate the movement of IAA across the plasma membrane (Marchant et al., 1999; Kramer and Bennett, 2006; Yang et al., 2006; Kerr and Bennett, 2007). IAA exits the cytoplasm via membrane-bound efflux carriers of the PIN-FORMED (PIN) family of proteins, which localize

asymmetrically on the plasma membranes of auxin-transporting cells, correlated with the direction of auxin flow (Blilou et al., 2005; Kerr and Bennett, 2007; Petrasek et al., 2006; Teale et al., 2006). The PIN proteins occupy a central role in the polar auxin transport complex (Friml and Palme, 2002). Full-length PIN proteins, especially PIN1, are continuously cycling between the plasma membrane and endosomal compartments (Geldner et al., 2001). Their polarity can be modulated rapidly in response to developmental or external cues, thus redirecting auxin. The rate, polarity, and symmetry of the flow of auxin is determined by the polar cellular localization of PIN auxin efflux carriers (Santelia et al., 2008). The differential growth response associated with gravitropism occurs in the elongation zone of root by the change of asymmetric distribution of auxin to the lower side of epidermal cells (Moore, 2002; Muday, 2001; Chen et al., 1998). In the tissue accumulating auxin, cell elongation is inhibited and the root tip bends downwards. This cell-to-cell or polar auxin transport is determined by asymmetric cellular localization of auxin in- and efflux components (Vieten et al., 2007; Wisniewska et al., 2006; Geisler and Murphy, 2006; Kerr and Bennett, 2007).

1-naphythylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) have been used extensively as synthetic auxin transport inhibitors (Rubery, 1990). Although both compounds inhibit polar auxin transport by inhibition of auxin efflux, they have different membrane binding features (Hertel et al., 1972; Katekar and Geissler, 1977; Brunn et al., 1992). NPA does not compete with IAA for a common binding site, whereas TIBA competes for binding. Naturally occurring plant endogenous flavonoids also show auxin transport inhibition activities (Jacobs and Rubery, 1988). A group of flavonoids including quercetin, apigenin, and kaempferol were observed to compete with NPA for binding to its receptor and consequently perturbed auxin transport. The regulatory function of flavonoids on auxin transport was tested in vivo in

Arabidopsis by comparing wild-type and a *transparent testa* (*tt4*) mutant with a mutation in the gene encoding the first enzyme in flavonoid biosynthesis, chalcone synthase (Brown et al., 2001). Flavonoids have been shown to modulate auxin transport in addition to auxin-dependent tropic responses (Peer and Murphy, 2007). Several targets of flavonoids that are involved in auxin transport mechanism have been proposed (Peer et al., 2004; Peer and Murphy, 2007). In addition to the PIN proteins, the P-glycoprotein (PGP)-type ATP-binding cassette (ABC) transporters, which hydrolyses ATP to transport substrates, have shown interactions with flavonols (Geisler et al., 2005; Lewis et al., 2007; Wu et al., 2007). ABC transporter families are known as the component involved with the membrane transport of endogeneous secondary metabolites in plants (Yazaki, 2006). Similar chemical structures between both synthetic and plant natural auxin transport inhibitors imply binding affinity of these compounds with auxin transporter components (Figure 1.4). Structure-activity complimentarity of auxin transport inhibitors have been described by Bures et al. (1991). They require an acidic functionality (e.g., carboxylic acid, acidic hydroxyl) attached to a tertiary, quaternary, or aromatic center. Among the GSs, indolyl and aromatic GSs may have the potential to interact with the auxin transporter components, since they have indolyl and aromatic side chains sharing the structural similarity with existing inhibitors. Elucidation of potential interactions of indolyl and aromatic GSs with the auxin transport components may help to characterize possible transport mechanisms for GSs. If indolyl and aromatic GSs interact with the known auxin transporter proteins, GSs may utilize component of the auxin transporter system for intra- and extracellular transport and translocation by direct or indirect interaction.

Table 1.1. GSs identified in broccoli, their common names, and parental amino acids from which they are derived.

Systematic name	Trivial name	Parental amino acid
3-methylthiopropy	Glucoiberverin	Methionine
3-methylsulphinylpropyl	Glucoiberin	Methionine
2-propenyl	Sinigrin	methionine
4-methylthiobutyl	Glucoerucin	methionine
4-methylsulphinylbutyl	Glucoraphanin	methionine
3-butenyl	Gluconapin	methionine
(2R) 2-hydroxy-3-butenyl	Progoitrin	methionine
(2S) 2-hydroxy-3-butenyl	Epiprogoitrin	methionine
5-methhylthiopentyl	Glucoberterion	methionine
5-methylsulphinylpentyl	Glucoalyssin	methionine
4-pentenyl	Glucobrassicanapin	methionine
2-hydroxy-4-pentenyl	Napoleiferin	methionine
3-indolymethyl	Glucobrassicin	tryptophan
N-methoxy-3-indolylmethyl	Neoglucobrassicin	tryptophan
4-methoxy-3-indolymethyl	4-Methoxyglucobrassicin	tryptophan
4-hydroxy-3-indolylmethyl	4-Hydroxyglucobrassicin	tryptophan
2-phenylethyl	Gluconasturtiin	phenylalanine

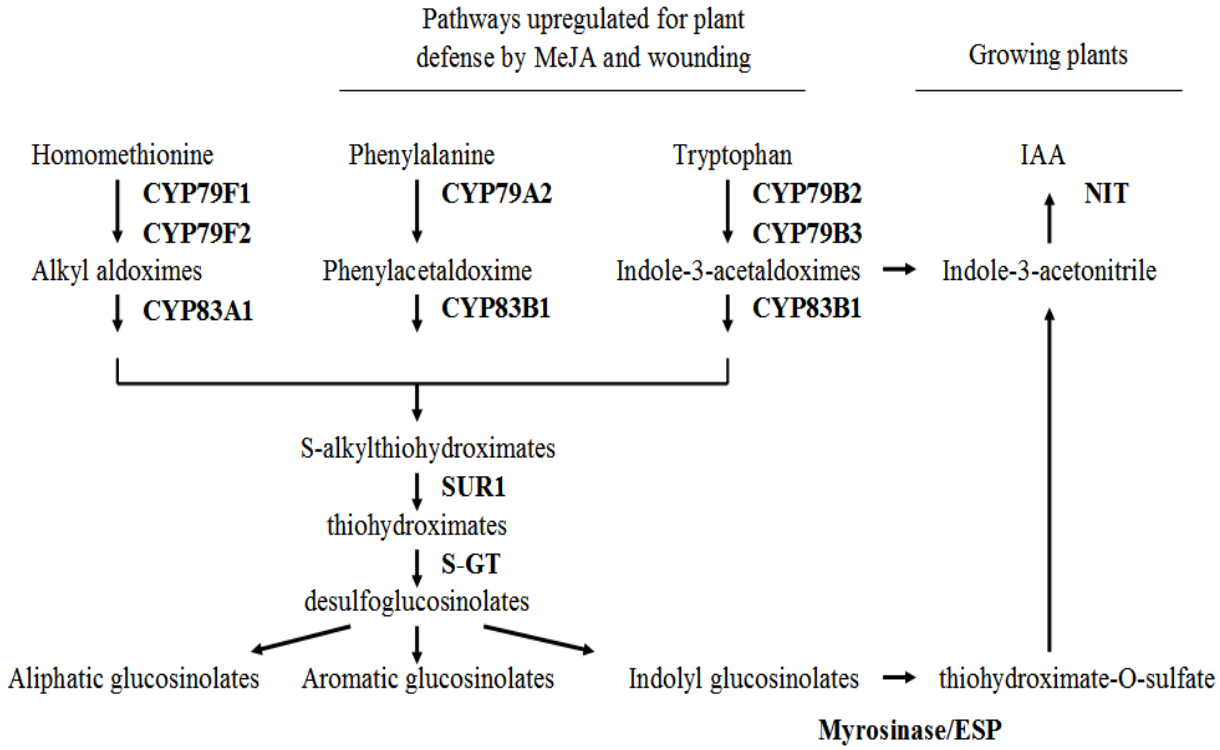


Figure 1.1. Biosynthetic pathways of aliphatic, indolyl and aromatic GSs. Enzymes associated with the each step of the biochemical pathway are indicated in bold.

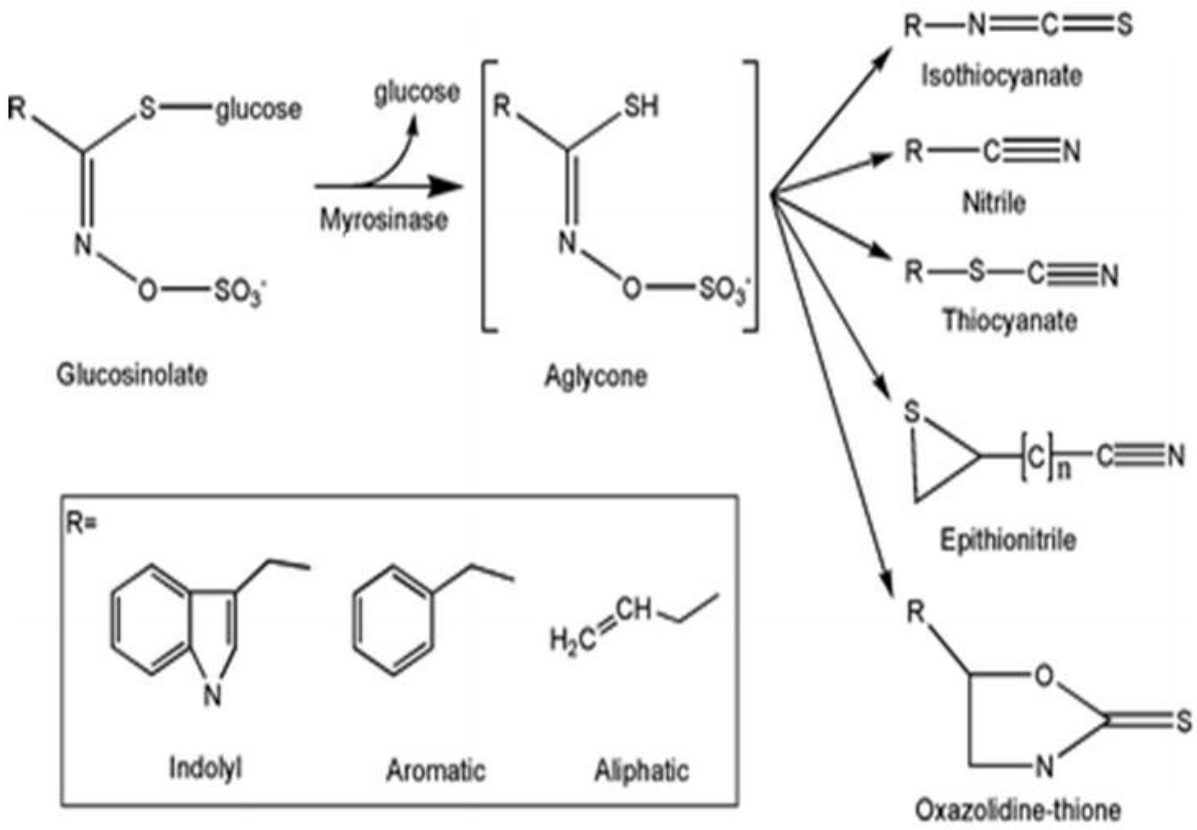


Figure 1.2. Glucosinolate structure and myrosinase catalyzed hydrolysis products. [Figure source: Yan and Chen. 2007]

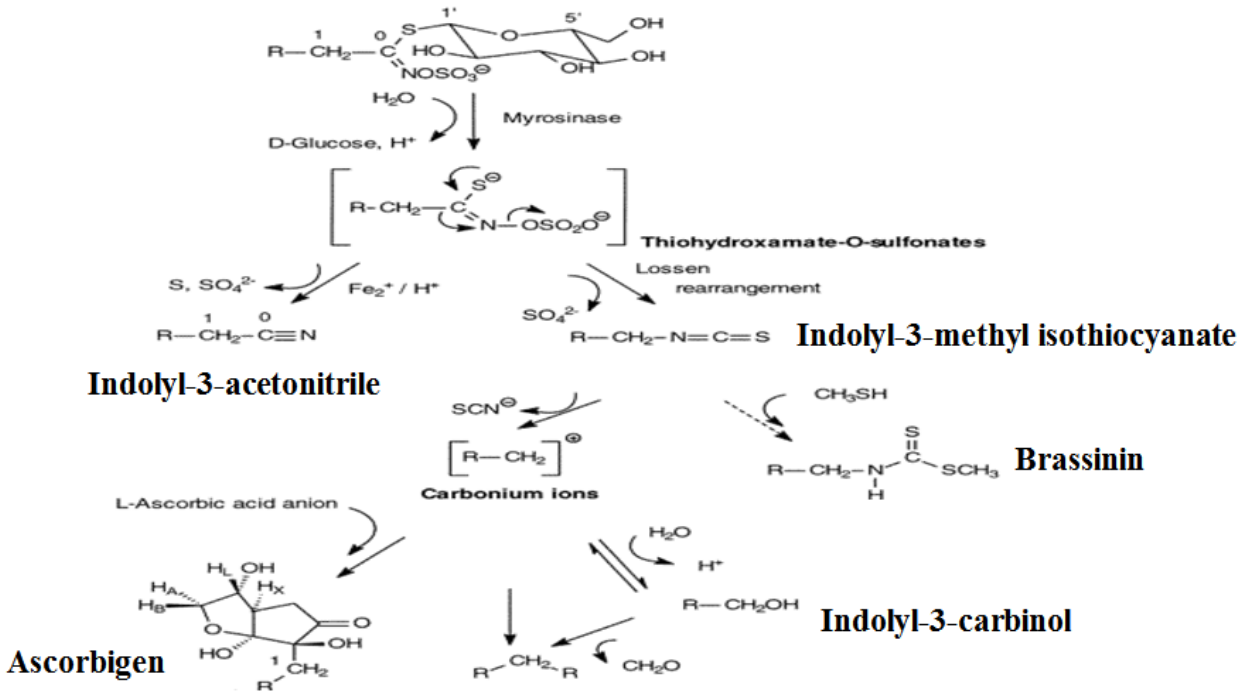


Figure 1.3. Products of the myrosinase-catalyzed hydrolysis of indolyl GSs. [Figure source: Agerbirk et al. 1998]

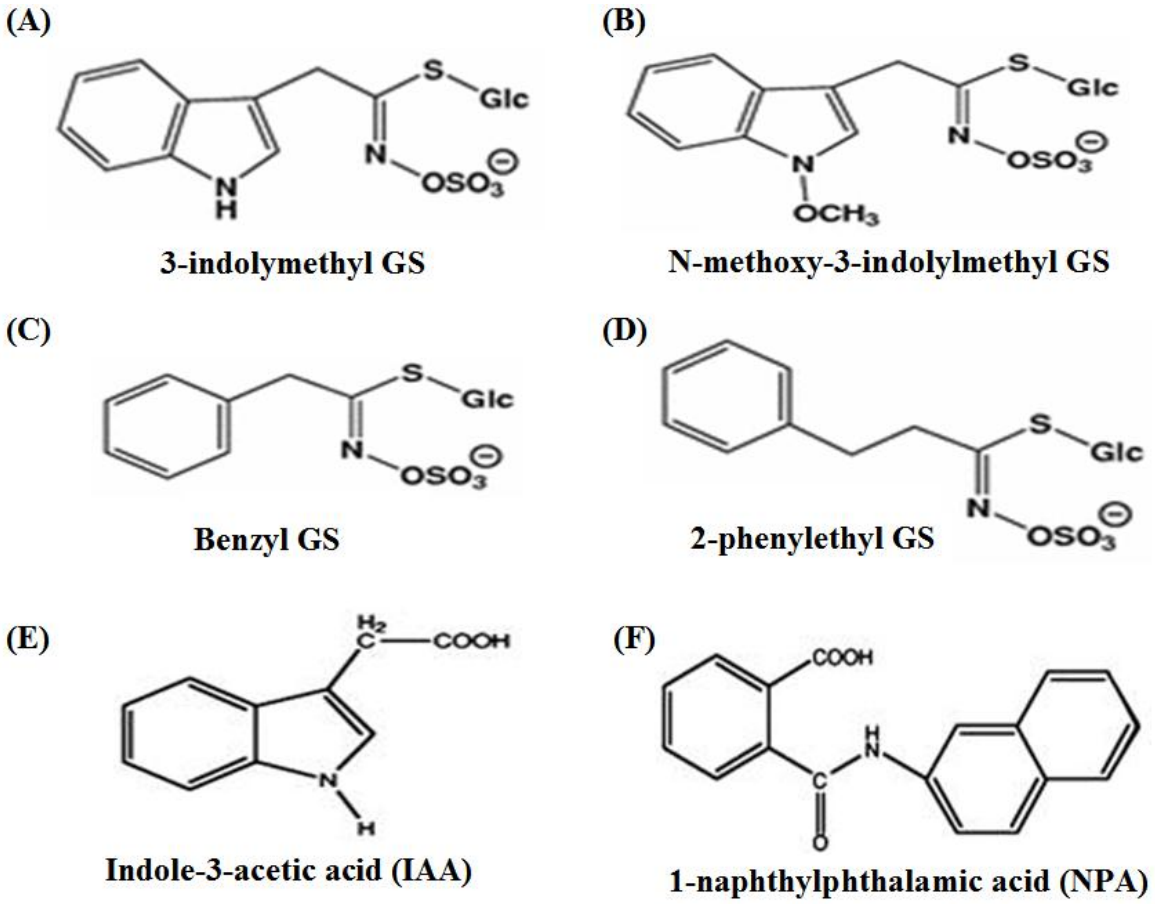


Figure 1.4. Chemical structures of indolyl and aromatic GSs (A, B, C and D), IAA (E), and NPA (F). [Figure source: Textor and Gershenzon, 2009]

CHAPTER 2

Changes in glucosinolate metabolism mediated by methyl jasmonate signaling in broccoli (*Brassica oleracea* L. ssp *Italica*)

2.1. Abstract

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is a rich source of glucosinolates (GSs), phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Among the various types of GSs, indolyl GSs are of particular interest due to the dramatic changes in the amounts of these compounds induced in response to treatments with methyl jasmonate (MeJA). Four genes encoding cytochrome P450 enzymes (CYP), CYP79B2, CYP79F1, CYP83A1, and CYP83B1 were isolated from broccoli using primers based on corresponding *Arabidopsis* and *Brassica rapa* gene sequences. Changes in GS accumulation and gene expression levels in response to MeJA treatment were analyzed in different tissue types at different developmental stages of broccoli. Greater accumulation of GSs with MeJA treatment was observed in apical leaves of broccoli seedlings and florets at harvest maturity. Increases in indolyl GS in apical leaves of seedlings and florets were coupled with the up-regulation of indolyl GS biosynthesis genes. These results suggest that the accumulation of indolyl GSs may be modulated by MeJA treatment in an organ-specific manner for optimal distribution of defense substances in the plant. The organ-specific accumulation of indolyl GSs may be coordinated by MeJA signaling for allocation of precursors required for *de novo* biosynthesis of GSs in source tissues and for translocation of indoly GSs from source to sink tissues.

2.2. Introduction

Glucosinolates, nitrogen- and sulfur-containing phytochemicals, are biologically active secondary metabolites primarily found in the family Brassicaeae (Fahey et al., 2001). GSs have a

common structure comprising a sulfonated oxime and a β-thioglucose residue, and a variable side chain of modified amino acids which is responsible for their chemical diversity (Wittstock and Halkier, 2002). GSs are hydrolized by the plant endogeneous enzyme myrosinase into nitrile, isothiocyanate, or thiocyanate forms, and these hydrolysis products have different biological activities, such as plant defense functions against generalist herbivores and pathogens, and human anti-carcinogenic bioactivity (Kirkegaard and Sarwar, 1998; van Poppel et al., 1999; Mithen et al., 2000; Noret et al., 2005). The roles of the GS hydrolysis products have received considerable attention due to their potentials as human cancer-prevention agents, crop protection compounds, and biofumigants in agriculture (Halkier and Gershenzon, 2006).

Jasmonic acid (JA) and methyl jasmonate (MeJA), a volatile methylester of jasmonic acid are plant signaling molecules responding to abiotic and biotic stress. GSs are a class of metabolites modulated by the jasmonate signaling pathway. GSs are present constitutively in plant tissues, but are also rapidly induced to higher levels by stress such as wounding, pathogen and herbivore attack (Bartlet et al., 1999; Kim and Jander, 2007; Bednarek et al., 2009). Application of exogeneous JAs has been known to induce the production of GS in *Brassica* species, particularly indolyl GS (Bodnaryk, 1994; Doughty et al., 1995; Kliebenstein et al., 2002; van Dam et al., 2003). JAs or wounding treatments increased expression of the tryptophane-metabolizing genes *CYP79B2* and *CYP79B3* as well as transcription factors *IQD1* and *AtDof1.1* leading to upregulation of genes related with the indolyl GS biosynthesis, and consequent accumulation in *Arabidopsis* (Mikkelsen et al., 2003; Levy et al., 2005; Skirycz et al., 2006).

Differential distribution of GSs in different plant organs and compositional changes occurring during development can provide information to understand how the plant regulates accumulation and distribution of these compounds. Significant differences in GS concentration

and composition were noted among various organs and developmental stages of *Arabidopsis* (Petersen et al., 2002; Brown et al., 2003). Germinating seeds had the highest total GS concentration, followed by young leaves, old leaves and roots. With transition from the vegetative to the reproductive stage, total GS concentrations decreased in leaves but increased in inflorescences and siliques (Brown et al., 2003). These patterns are similar to those found in other glucosinolate-containing species (Booth et al., 1991; Fahey et al., 2001). These compositional features indicate that GS accumulation tends to be concentrated into plant organs associated with plant survival and reproduction such as the seeds, sprouts and the inflorescence. It has been hypothesized that these organ-specific distributions are associated with optimal distribution of defense substances to protect against insects and other herbivores (Zangerl and Bazzaz, 1993). For instance, the proportionally dominant 2-phenylethyl GS (aromatic GS) in *Brassica napus* root was associated with the susceptibility of the crop to the root lesion nematode (*Pratylenchus neglectus*) (Potter et al., 2000).

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is a rich source of GSs, phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Broccoli also has been shown to be a good source of several health promoting phytochemical compounds including carotenoids, tocopherols, and ascorbic acid (Ibrahim and Juvik, 2009). With these health-promoting properties, broccoli is a good model system for studying GS metabolism in a crop plant, due to the abundance of various GSs, ease of sampling various tissues during different developmental stages, and distinct morphological characteristics of the enlarged inflorescence (immature florets) with abundant GS accumulation. In the same taxonomic family, broccoli shares genetic homology with *Arabidopsis* (Lan et al., 2000), which also contains GSs

and provides a resource to isolate genes and conduct comparative gene functional studies related with GS metabolism.

The aim of this study was to analyze changes in indolyl GS accumulations modulated by MeJA treatment in broccoli. Application of exogenous MeJA or mechanical wounding coupled with GS concentration quantification focusing on compositional and distributional changes. *Arabidopsis* homologous genes involved with the biosynthesis of GSs (*CYP79B2, CYP79F1, CYP83A1* and *CYP83B1*) were isolated from broccoli using primer sets based on *Arabidopsis* homologous sequences, and relative expression levels of these and two other genes (myrosinase and epithiospecifier protein), involved in the hydrolysis of GSs, were analyzed by quantitative RT-PCR (qRT-PCR) with and without MeJA treatment.

2.3. Materials and methods

2.3.1. Plant material

The effects of MeJA or wounding treatments on GS accumulation was studied in different tissue types at two developmental stages (seedlings and plants at harvest maturity) in two commercial hybrid broccoli cultivars 'Pirate' (Asgrow Seed Co.) and 'Green Magic' (Sakata Seed Co.). Seeds of each broccoli genotype were germinated in small pots filled with sunshine® LC1 professional soil mix. Seedlings were transplanted into 5 cm pots and grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25℃/15℃ and 14-h/10-h day/night temperature regime and with supplemental light. Experimental design was a randomized complete block with three replicates of 3 seedling plants. For the mature broccoli plant experiments, seedlings were transplanted into 22.8 cm pots and grown under the same greenhouse conditions as described above. Three weeks after transplanting, plants were fertilized with a solution of 20N-20P-20K twice a week. Experimental design was also a randomized

complete block with three replicates of 3 plants spaced 30 cm apart with 30 cm between rows on greenhouse benches.

2.3.2. MeJA and wounding treatments

Six week old broccoli seedlings and broccoli plants at harvest maturity were subjected to the MeJA treatment. All aerial parts of the plant were sprayed with 250 µM MeJA (Sigma-aldrich, St. Louis, MO) containing 0.1% Triton X-100 (Sigma-aldrich, St. Louis, MO) until plant surfaces are fully saturated with MeJA solution. Control plants were sprayed with water containing 0.1% Triton X-100. Plants were spray treated in isolation and allowed to dry prior to being placed back onto greenhouse benches. For wounding treatments, the fourth and fifth basal leaf from the shoot apex of six-week old seedlings were subjected to a wounding device designed to pierce each basal leaf with 234 (18 x 13) small nails mounted on a wood block (15 cm x 13 cm) which generated approximately 60-80 perforations per treated leaf.

Different tissue samples from broccoli seedlings and mature plants were harvested at 1, 2 and 4 days after treatments. In a six-week old seedlings, samples were harvested from apical (the first, second and third apical leaf from the shoot apex) and basal (4th-6th leaf) leaves, petioles of basal leaves, and stem tissues. In mature broccoli plants, immature florets, apical (1st- 4th leaf nodes below the florets excluding small leaves less than 3 cm of leaf diameter) and basal (8th-9th leaf nodes) leaves, and stem (from below floret to 9th leaf node) were harvested. Three plant subsamples were bulked in each of three replicates, frozen in liquid nitrogen, ground into powder, and stored at -80 ℃ for RNA extraction and a portion lyophilized and stored at -20℃ until HPLC analysis.

2.3.3. RNA extraction, RT-PCR, cloning, and sequencing

Total RNA was extracted from control and MeJA treated leaf tissues using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The first strand of cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase with oligo-(dT) primer according to the instructions of the Reverse Transcription System (Promega, Madison, WI). Using known *Arabidopsis* and *Brassica rapa* gene sequence information, the primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3) to isolate four broccoli homologous cytochrome *P450* genes, *CYP79B2*, *CYP79F1*, *CYP83A1* and *CYP83B1,* known to be associated with GS biosynthesis. PCR amplification was performed using the GoTaq® PCR Core System (Promega, Madison, WI) following the protocol described by the manufacturer. The amplified PCR products were cloned with pGEM® -T Easy Vector System (Promega), and the clones were sequenced in the W. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using the ABI 3730XL Capillary Sequencer (Applied Biosystems, Foster City, CA). The amino acid sequences deduced from the isolated cDNA sequences were subjected to phylogenetic tree analysis using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

2.3.4. Gene expression

Quantitative real-time PCR (qRT-PCR) was performed using the SYBR® Green PCR Master Mix (Applied Biosystems) with the ABI PRISM/Taqman 7700 Sequence Detection System (Applied Biosystems). Total RNA was extracted using the RNeasy Mini Kit with RNase-Free DNase set (QIAGEN), and cDNA was synthesized from 1 μg of the total RNA using the Superscript® III First-Strand Synthesis System with the mixture of random hexamer and oligo-(dT) primers (Invitrogen, Carlsbad, CA). Target genes and primer sequences are listed in Supplementary Table S2.1. Broccoli *Actin-1* was used as the normalizer (reference) gene. All

reactions were run in triplicate from the corresponding three biological replications, and ratio calculations were conducted by the mathematical model proposed by Pfaffl (2001) using ΔCt (the cycle threshold value difference between control and sample of target and reference gene transcript).

Northern blot analysis was performed with the total RNA isolated by Trizol reagent (Invitrogen). 20 μg of the total RNA (per lane) was separated in formaldehyde-agarose gels, and RNA transferred onto Hybond-XP nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were hybridized with a ^{32}P-labeled probe, and washed at 60 ℃ for 10 min in 2 × SSC, 10 min in 1 × SSC, and 15 min in 0.5 × SSC with 0.1% (w/v) SDS.

2.3.5. Glucosinolate analysis

GSs in lyophilized tissues were extracted and analyzed by high-performance liquid chromatography using a reverse phase C^{18} column as described by Kim and Juvik (submitted). GSs were desulfated with sulfatase solution (Sigma-Aldrich, St. Louis, MO) in columns containing DEAE Sephadex A-25 resin (Sigma-Aldrich, St. Louis, MO), and eluted desulfo-GSs were separated on a HPLC system consisting of a DIONEX GP40 gradient pump, with a AD20 variable UV detector set at 229 nm wavelength, auto-sampler, all-guard ™ cartridge precolumn (Alltech, Lexington, Kentucky), and a Lichosphere 100 RP-18 column (Grace Co. Deerfield, IL). The type and amount of GSs in each sample were calculated in comparison to certified glucosinolate levels in a standard rapeseed reference material (BCR 367, Commission of the European Community Bureau of References, Brussels, Belgium). GSs were quantified with benzylglucosinolate (POS Pilot Plant Corp, Saskatoon, SK, Canada) as an internal standard using UV response factors for different types of GSs determined by Wathelet et al., (2001). The identification of intact and desulfo-GS profiles were validated by LC-tandom MS using a Waters

QT of Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC-MS using a Finnigan LCQ Deca XP, respectively. The molecular ion and fragmentation patterns of individual intact and desulfo GS were matched with the literature for GS identification (Tian et al., 2005; Barbieri et al., 2008).

2.3.6. Statistical analysis

GS concentrations obtained from different broccoli tissues treated with or without MeJA were analyzed by the GLM procedure and Fisher's LSD tests using the SAS statistical analysis package (SAS version 8.0).

2.4. Results

2.4.1. Isolation of cDNA encoding CYP79B2, CYP79F1, CYP83A1 and CYP83B1 from broccoli

Genes encoding cytochrome P450 enzymes (CYP), CYP79B2 and CYP79F1, catalyzing the conversion of amino acids to aldoximes leading to indolyl and aliphatic GS, respectively, and CYP83A1 and CYP83B1, which convert aldoximes to thiohydroximic acids (reviewed in Halkier and Gershenzon, 2006; Sønderby et al., 2010) were selected as the target genes to be used as the indicators for analysis of expression changes in GS biosynthesis after MeJA treatments. The corresponding genes in broccoli were successfully amplified by PCR reactions using primers based on *Arabidopsis* and *Brassica rapa* homologous gene sequences. The amino acid sequences deduced from the isolated broccoli gene sequences corresponded to the *Arabidopsis* protein, CYP79B2, CYP83B1, CYP79F1 and CYP83A1 by 100, 100, 92 and 94%, respectively, with overall 92% amino acid sequence similarity. The phylogenetic tree generated from the aligned amino acid sequence of broccoli with the two reference sequences from *Arabidopsis* and *Brassica rapa* demonstrated that the phylogenetic groups separated depending

on their cytochrome P450 subfamily class and type (Figure 2.1). The isolated broccoli *P450* genes share homologous protein motifs with *Arabidopsis* GS biosynthesis genes, which suggests similar gene product function in broccoli.

2.4.2. Gene expression in different tissues at different developmental stages

Relative expression of the four broccoli *P450* genes, two other genes involved in the hydrolysis of GSs [*Brassica oleracea* myrosinase (*BoMYO*) and epithiospecifier (*BoESP*) protein gene], and two genes [*Brassica oleracea* defensin (*BoDEF*) and pathogenesis-related (*BoPR*) gene] known to be positive indicators of MeJA signaling pathway (Mikkelsen et al., 2003) were analyzed in apical and basal leaves and stems of six weeks old seedlings, and in floret tissue of mature broccoli cultivar 'Green Magic' by qRT-PCR with or without MeJA treatment (Figure 2.2). The four broccoli *P450* genes differentially responded to MeJA treatment with up-regulation of *BoCYP79B2* (over 52-fold), *BoCYP83B1* (over 20-fold), and *BoCYP83A1* transcripts (over 5-fold) in both apical and basal leaves of seedlings. Levels of maximum expression in these three transcripts was observed at 1 day after MeJA treatment and thereafter declined in seedling apical and basal leaf tissues. Florets of mature plants displayed less dramatic increases in *BoCYP79B2* (12-fold), *BoCYP83B1* (4-fold), and *BoCYP83A1* (5-fold) expression compared to those levels observed in seedling leaf tissues (Figure 2.2). In seedling stem tissue, *BoCYP79B2* and *BoCYP83A1* expression was increased 5 fold after MeJA treatment. *BoCYP79F1* did not show consistent trends of up-regulation in response to MeJA treatment across all broccoli tissues tested in our experiment, suggesting limited interaction with MeJA signaling. MeJA treatment resulted in over 3-fold increases of *BoESP* expression in both apical and basal leaves, and 7-fold increases of *BoMYO* in stems of seedlings after MeJA treatment. As

anticipated, expression of *BoDEF* and *BoPR*, positive indicators of MeJA signaling, were up-regulated in our experiment (Figure 2.2 and Supplementary Table S2.2).

Northern blot analysis also revealed tissue specific up-regulation of the four broccoli *P450* genes in apical and basal leaves, petioles, and stems of six-week old seedlings, and immature florets, apical and basal leaves, and stems of mature broccoli plants with MeJA treatment (Figure 2.3). Similar to results obtained from qRT-PCR, *BoCYP79B2* exhibited overall higher levels of expression in MeJA treated tissues compared to other broccoli *P450* genes. Apical leaves of seedlings showed the highest expression of *BoCYP79B2* after MeJA treatment compared to other tissues in seedlings and plants at harvest maturity. Petioles of seedlings and basal leaves and stems of mature broccoli plants did not show a significant response to MeJA treament. The dramatic up-regulation of genes associated with indolyl GS biosysthesis in response to MeJA treatment was primarly observed in young and developing sink tissues such as apical leaves of seedlings and in florets and apical leaves of mature broccoli plants. Expression of the other two genes, *BoCYP79F1* and *BoCYP83A1*, was not significantly affected by MeJA treatment in seedlings and mature broccoli plants.

2.4.3. MeJA modulated changes in aliphatic and total GSs

Application of exogeneous MeJA to broccoli seedlings and mature plants significantly increased total GS concentrations in all tissues tested in our experiment (Figure 2.4 and Supplementary Table S2.3-8). MeJA-mediated increase in indolyl GS concentration altered the proportion of GSs making them the dominant forms in apical and basal leaves of seedlings and florets of mature broccoli plants. Apical leaves of the seedlings demonstrated the highest fold increase of total GS concentrations in response to MeJA treatment (5.3- and 2.6-fold in ‘Green Magic’ and ‘Pirate’, respectively), while total GS concentrations increased by 1.9- and 2.1-fold

in basal leaves of these two genotypes. In mature broccoli plants, increases in total GS concentrations were shown in floret and young apical leaf tissues (Figure 2.4). Floret tissues displayed the highest total concentrations of GSs (26.3 and 34.7 μM/g of dry weight in 'Green Magic' and 'Pirate', respectively) among the tissues of seedlings and mature plants subjected to MeJA treatment. Significant increases in the aromatic GS, 2-phenylethyl GS concentrations were observed in florets and apical leaves of mature plants after MeJA treatment. Aliphatic GSs including 4-methylsulfinylbutyl and 3-butenyl GS showed no consistent response to MeJA treatment (Supplementary Table S2.3-8). Petioles and stems of seedlings and stems of mature plants also responded to MeJA treatment with the significant increases in total GS concentrations primarily contributed by increased levels of indolyl GSs. Up-regulation of the *BoCYP79B2* gene in stem tissues of broccoli seedlings after MeJA treatment suggests it contains cells capable of GS biosynthesis. Wounding treatments in two basal leaves of seedlings were less effective to increase GS accumulation than application of exogeneous MeJA into whole aerial portions of the plant. However, significant increases in total GS concentrations were observed in apical leaf not subjected to wounding, indicating a systemic response for GS accumulation (Figure 2.4).

2.4.4. Genotype-dependent differential response to MeJA

While the concentration of indolyl GSs significantly increased in all tissues of seedlings and mature plants tested, 3-indolylmethyl and N-methoxy-3-indolylmethyl GS differentially responded to MeJA treatment in the two broccoli cultivars, 'Green Magic' and 'Pirate' (Figure 2.5). Greater response in the concentration of N-methoxy-3-indolylmethyl GS was observed in all tissue types in 'Green Magic' in response to MeJA. In contrast, 'Pirate' exhibited greater increases of 3-indolylmethyl GS rather than N-methoxy-3-indolylmethyl GS after MeJA treatment. Accumulation of 3-indolylmethyl GS was also the dominant indolyl GS observed in

floret tissue of VI-158 in response to MeJA. These results suggests genetic variation for differential induction of the two different indolyl GS forms may exist between broccoli genotypes via methoxylation at N(1) position of the indole ring. Recently, one of *P450* genes, *CYP81F2*, was identified as the *Indole Glucosinolate Modifier1* (*IGM1*) gene through QTL fine-mapping using genetic variation in indolyl GSs in *Arabidopsis*. The function of CYP81F2 catalyzing conversion of 3-indolylmethyl to 4-hydroxy-3-indolylmethyl GS was verified by analysis of *Arabidopsis* T-DNA knockout mutants (Pfalz et al., 2009). This result indicates that variation in indolyl GS forms in different genotypes are generated by the corresponding gene expression.

Accumulation patterns of indolyl GSs also varied across genotypes after wounding treatments. 'Pirate' seedlings exhibited a greater reduction in indolyl GSs in both apical and basal leaves of seedlings 4 days after wounding (Figure 2.5 B). Reduction in indolyl GS concentrations after wounding may be the consequence of hydrolysis from the mixing of myrosinase with indolyl GS. Differential reductions in indolyl GS concentrations after wounding across genotypes may also be due to differences in myrosinase activity.

2.5. Discussion

The plant response mediated by MeJA provides a system to investigate how plants defend against biotic stress through the generation and distribution of indolyl GS compounds. MeJA-mediated indolyl GS induction may depend on size of available pools of precursors required for *de novo* biosynthesis of GS in the plant (Chapter 5). Under limited sulfur or sulfur-containing amino acid availability, artificially induced by selenium fertilization, MeJA-mediated indolyl GS accumulation was completely inhibited in broccoli seedlings, whereas biosynthesis

was maintained in floret tissue (Chapter 5). Compositional and distributional changes in GSs is organ-specific manner under conditions of biotic stress.

2.5.1. Organ specific accumulation of indolyl GSs mediated by MeJA treatment in broccoli

Our results suggest that the accumulation of indolyl GSs may be differentially modulated by MeJA treatment in an organ-specific manner for optimal distribution of compounds to provide defense against insect and other herbivores. In our preliminary experiments, application of MeJA to aerial portions of broccoli seedlings did not result in significant changes in GS concentrations in root tissue (Supplementary Table S2.9-10), consistent with a previous report in *Brassica napus* (van Dam et al., 2003). However, application of MeJA to roots of broccoli sprouts significantly increased the concentration of 2-phenylethyl GS in root tissue, indicating root-specific accumulation of 2-phenylethyl GS.

Differential changes in the concentrations of 3-indolylmethyl and N-methoxy-3-indolylmethyl GS, varying in different broccoli genotypes in response to MeJA treatment suggest that they may serve distinct biological roles in plant defense. 4-methoxy indolyl-3-methyl glucosinolate has been shown in *Arabidopsis* to respond to salicylic acid signaling for antifungal defense (Kliebenstein et al., 2002; Bednarek and Osbourn. 2009).

N-methoxy-3-indolylmethyl GS was the dominant form to increase in petioles and stems of broccoli seedlings across genotypes after MeJA treatment, suggesting that N-methoxy-3-indolylmethyl GS may act as the primary form of phloem-translocated GS.

2.6. Conclusions

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is a rich source of GSs, phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Epidemiological studies have demonstrated that increased consumption of cruciferous vegetables

in human diets is associated with a reduced risk of a number of cancers (Verhoeven et al., 1997), and these chemopreventive properties are mainly attributed to GS degradation products. The hydrolysis product of the 3-indolylmethyl GS, indole-3-carbinol, has proven to be successful in National Cancer Institute clinical trials against breast cancer (Telang et al., 1997) and respiratory papilloma (Rosen et al., 1998). *N*-methoxyindole-3-carbinol, the hydrolysis product of *N*-methoxyindolyl-3-GS (neoglucobrassicin), has also shown similar anticarcinogenic activities comparable to indole-3-carbinol (Neave et al., 2005; Stephensen et al., 2000). Increased accumulations of indolyl GS in broccoli florets may provide a value-added inducement for increased broccoli consumption with superior health promotion effects. In spite of the commercial importance of broccoli with its human health promoting properties, information on GS metabolism is incomplete in its understanding on how GSs accumulate and are translocated and distributed in an organ-specific manner. Our experimental results provide information on genes and their expression patterns associated with differential accumulation of indolyl GSs in different organs of broccoli. Selection and breeding of broccoli germplasm based on differential response of bioactive compounds to MeJA or wounding may be employed to enhance the levels of human health promoting compounds with potentially enhanced plant registance to broccoli pests and pathogens.

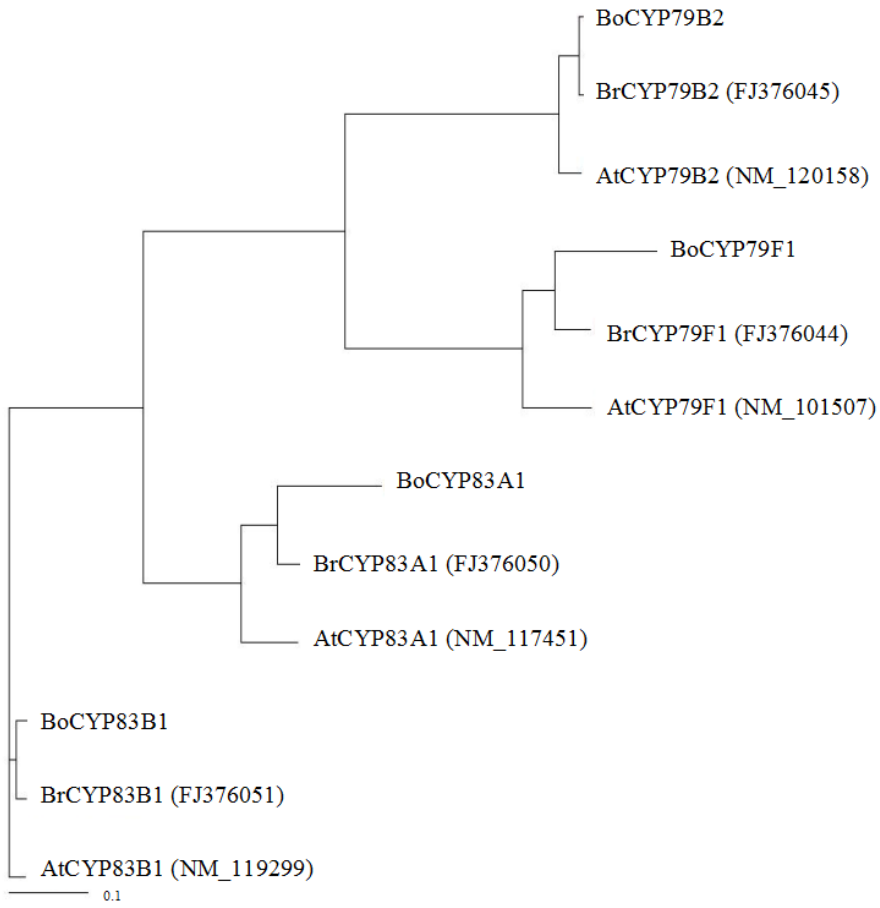


Figure 2.1. Phylogenetic tree of *Brassica* cytochrome P450s putatively associated with glucosinolate biosynthesis. Bo, Br and At indicate *Brassica oleracea*, *Brassica rapa* and *Arabidopsis thaliana*, respectively. The tree was constructed using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

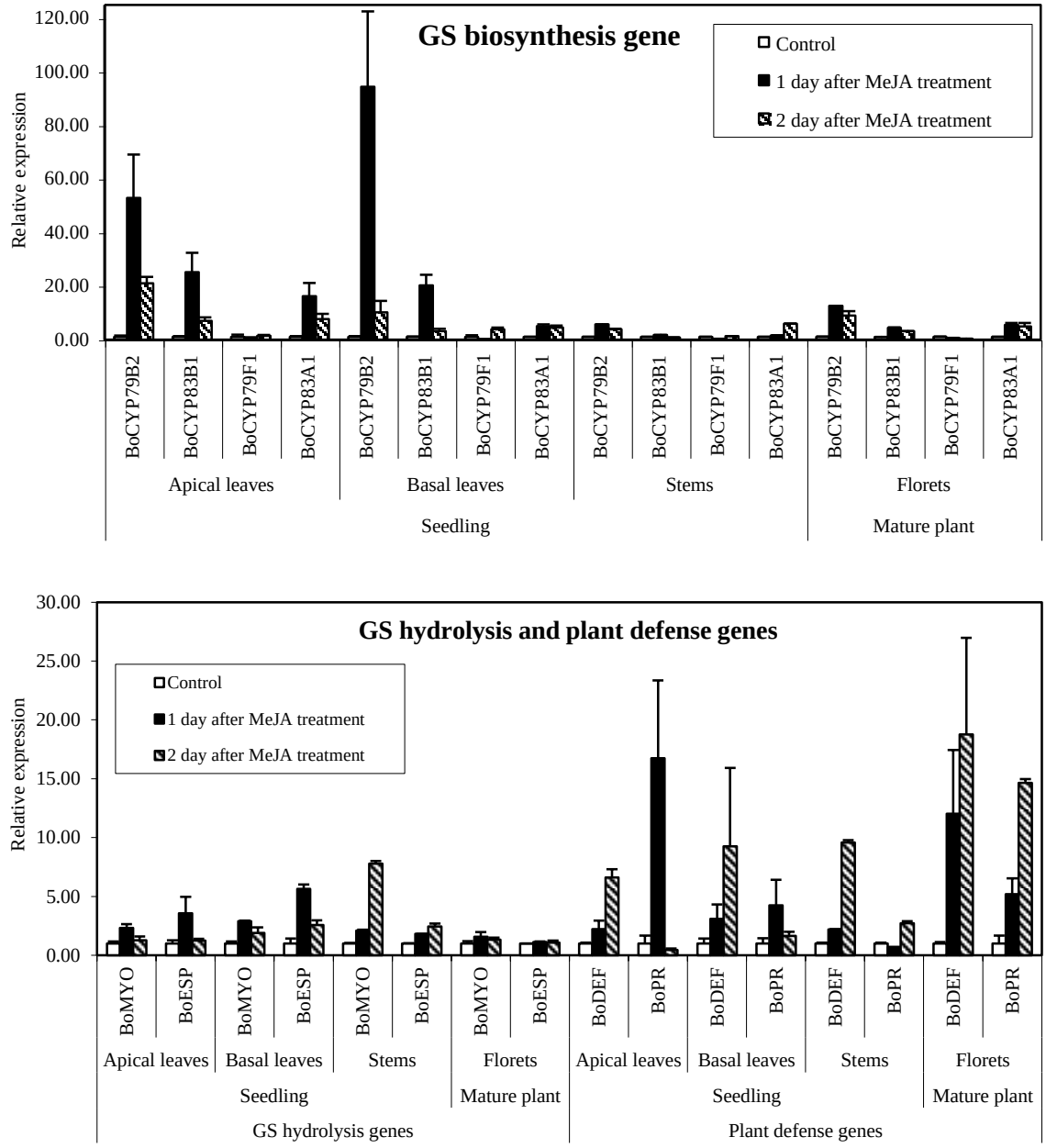


Figure 2.2. qRT-PCR analysis of MeJA-regulated expression of different genes involved in GS biosynthesis and hydrolysis, and plant defense response in apical and basal leaf and stem of six weeks old broccoli seedlings, and floret of mature broccoli plants with or without MeJA treatment. The transcript levels of genes were normalized with the *BoACT1* expression, and error bars represent standard error of means of triplicate biological replications.

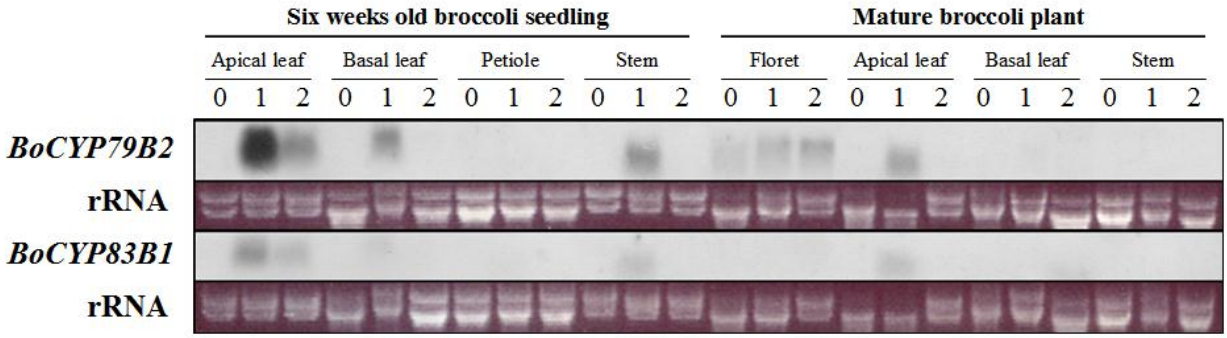


Figure 2.3. Tissue specific expression of *BoCYP79B2* and *BoCYP83B1* in broccoli 'Green Magic' with MeJA treatment. Northern blot analysis was performed on apical and basal leaf, petioles of basal leaves, and stems of six-week old seedlings, and florets, young apical leaves, mature basal leaves and stems of mature plants 1 and 2 days after treatment with MeJA. The bottom panels of each northern hybridization illustrates amounts of rRNA as loading controls.

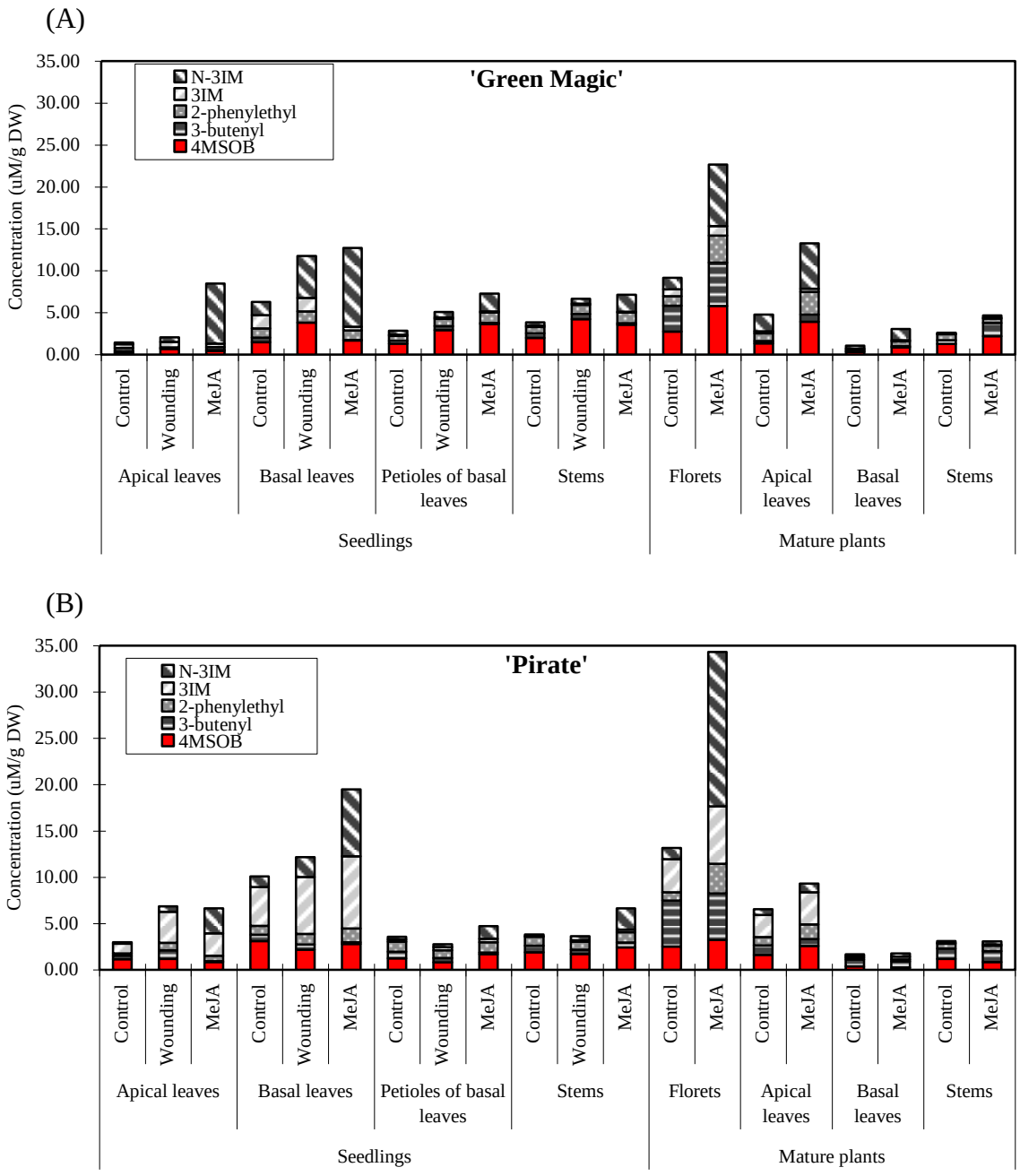


Figure 2.4. Changes in GS concentrations (μg/g DW) in apical and basal leaves, petioles of basal leaves, and stems of broccoli seedlings, and florets, apical and basal leaves and stems of mature plants in 'Green Magic' (A) and 'Pirate' (B) with wounding or MeJA treatments in basal leaves of seedlings. 4MSOB is an abbreviation of 4-methylsulphinylbutyl GS, 3IM of 3-indolymethyl GS, and N-3IM of N-methoxy-3-indolylmethyl GS.

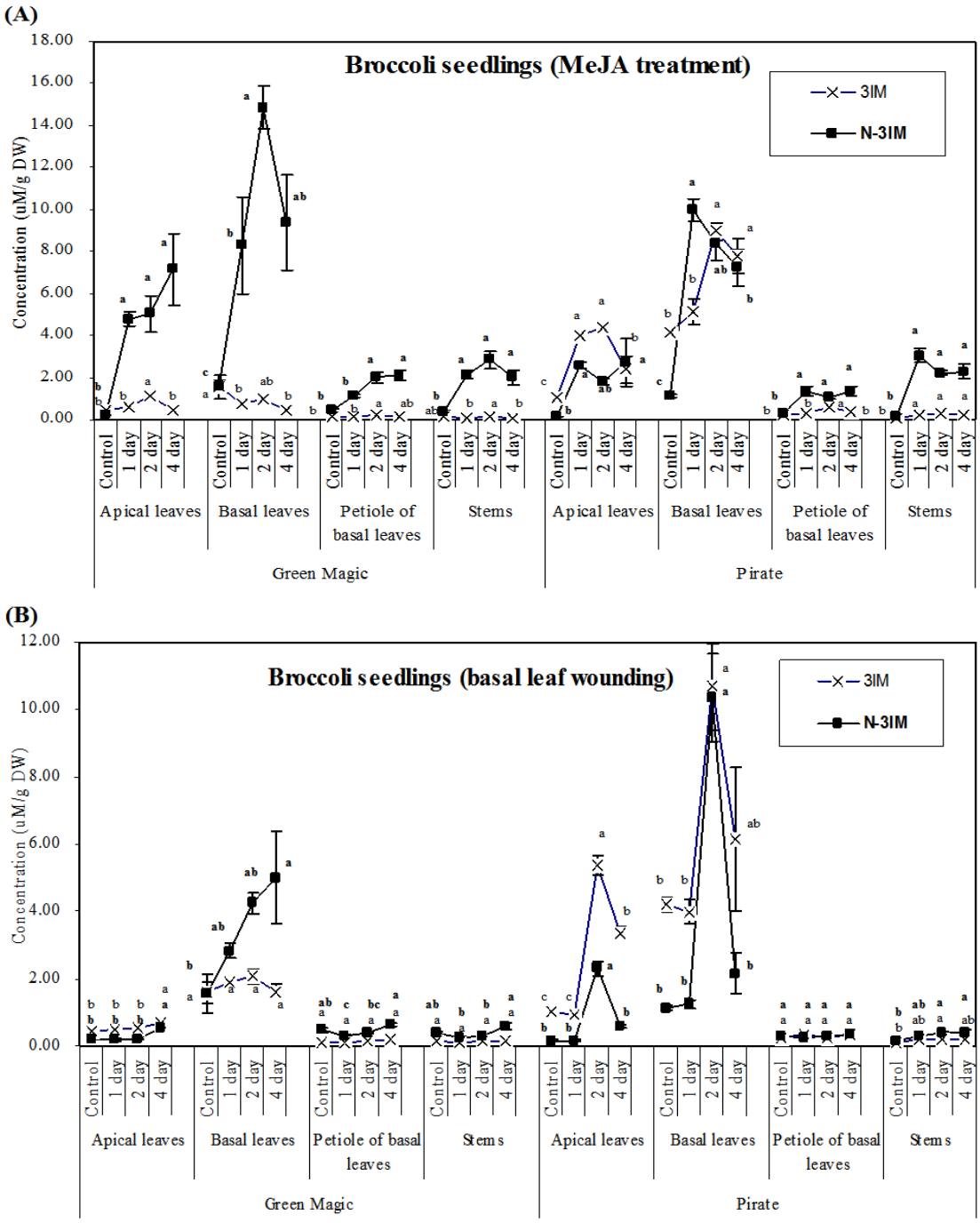


Figure 2.5. Changes in 3-indolymethyl GS and N-methoxy-3-indolylmethyl GS concentrations (μM/g DW) in different tissues of the broccoli seedlings with MeJA (A) and basal leaf wounding (B), and in mature broccoli plants with MeJA (C) at 0 (control), 1, 2, and 4 days after treatments. 3IM is an abbreviation of 3-indolymethyl GS, and N-3IM of N-methoxy-3-indolylmethyl GS. Means with a different letter are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis. Lines and letters for N-3IM are indicated in bold.

(C)

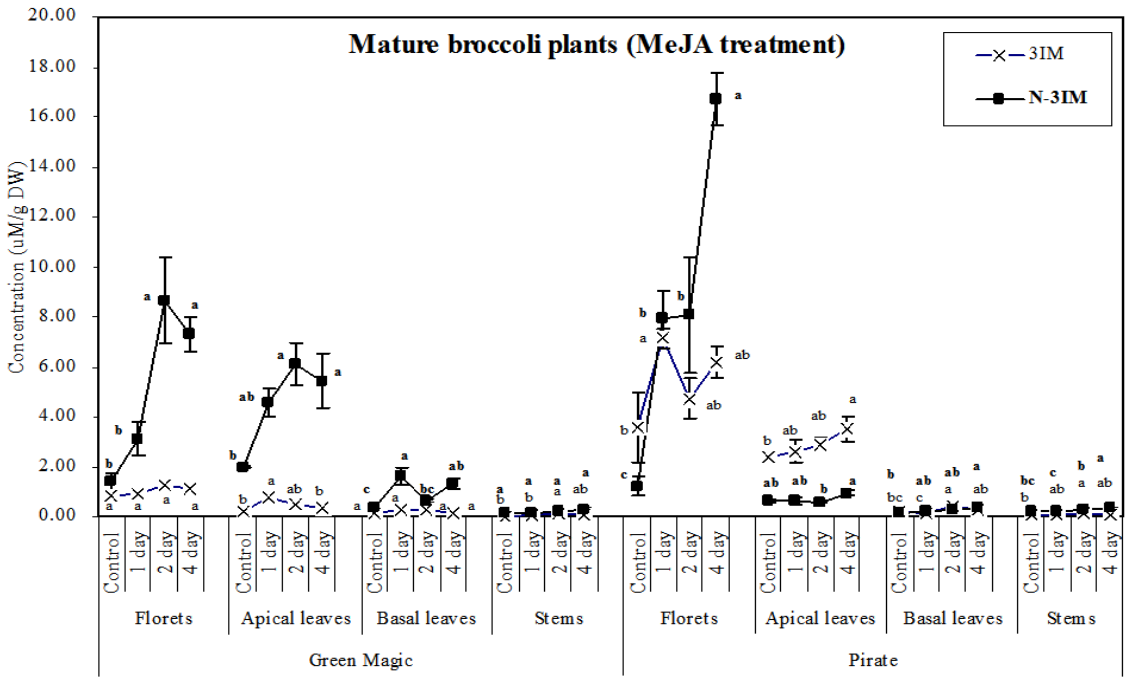


Figure 2.5. Continued.

Supplementary Table S2.1. List of primers used for qRT-PCR in broccoli.

Target gene (Accession number)	Description	Forward Primer (5'-3')	Reverse Primer (5'-3')
Glucosinolate biosynthesis			
BoCYP79B2	*Brassica oleracea* var. *italica* cytochrome P450 (CYP79B2)	AGCCAAGTCCTTCTCAGTCG	ACGAGATAAACCGGAGATCG
BoCYP83B1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP83B1)	ACGGAACCGAGATGAAGAGA	CTCTCTTGAGACGCGCACTA
BoCYP79F1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP79F1)	TCCGATGGTTCTCATGTTGA	AACCGGATATCGCATGTTTC
BoCYP83A1	*Brassica oleracea* var. *italica* cytochrome P450 (CYP83A1)	TCAAGACGCAAGACGTCAAC	CAAGTGGTTCATCCCCATCT
Glucosinolate hydrolysis			
BoMYO (EU004075)	*Brassica oleracea* myrosinase (MYO)	AACGCCTTTCGTTACCCTCT	TCACCTTTCCACCAAATTCC
BoESP (DQ059298)	*Brassica oleracea* var. *italica* epithiospecifier (ESP) protein	CGAGAAGCTCACATGGCATA	CTTGGACGGAGAGATTGACC
Plant defense			
BoDEF (EF423802)	*Brassica oleracea* var. *gemmifera* defensin (DEF)	GCCCTTGTTATTTTTGCTGCT	CACCATTGTTGGTGCTTCTG
BoPR (EF423806)	*Brassica oleracea* var. *gemmifera* pathogenesis-related (PR) protein	CCACCATTGTTACACCTTGCT	AACCTTTGGGTCAACGAGAA
qRT-PCR controls			
BoACT1 (AF044573)	*Brassica oleracea* actin (ACT1)	TCTCGATGGAAGAGCTGGTT	GATCCTTACCGAGGGAGGTT
Bo18S rRNA (AF513990)	*Brassica oleracea* 18S ribosomal RNA (18S rRNA)	ACGGGGAAACTTACCAGGTC	TTCCTCGTTGAAGACCAACA

Supplementary Table S2.2. Summary of qRT-PCR results obtained in apical and basal leaves and stems of six-week old broccoli seedlings, and florets of mature broccoli plants with or without MeJA treatment. The transcript levels of genes were normalized with the *BoACT1* expression, and presented as the relative expression (± SE of means of triplicate biological replications).

MeJA (days after treatment)	Seedlings									
	Apical leaves			Basal leaves				Stems		
	0	1	2	0	1	2	4	0	1	2
18S rRNA	1.00±0.12	0.45±0.10	1.76±0.05	1.00±0.12	0.73±9.14	2.42±0.85	1.19±0.06	ND	ND	ND
Glucosinolate biosynthesis										
BoCYP79B2	1.00±0.55	52.92±16.26	21.08±2.40	1.00±0.30	94.45±28.19	10.17±4.24	0.63±0.15	1.00±0.05	5.65±0.01	3.86±0.24
BoCYP83B1	1.00±0.36	25.19±7.20	7.06±1.29	1.00±0.24	20.29±3.87	3.19±0.89	0.29±0.03	1.00±0.05	1.70±0.05	0.82±0.07
BoCYP79F1	1.00±0.88	0.36±0.48	1.39±0.34	1.00±0.65	0.13±0.05	3.81±0.73	0.68±0.36	1.00±0.07	0.24±0.04	1.26±0.07
BoCYP83A1	1.00±0.27	16.24±4.86	7.62±2.06	1.00±0.15	5.05±0.70	4.52±0.73	0.67±0.08	1.00±0.07	1.58±0.03	5.91±0.15
Glucosinolate hydrolysis										
BoMYO	1.00±0.18	2.34±0.31	1.29±0.31	1.00±0.18	2.89±0.07	1.91±0.48	0.67±0.04	1.00±0.06	2.11±0.07	7.79±0.23
BoESP	1.00±0.29	3.58±1.39	1.27±0.14	1.00±0.44	5.65±0.37	2.60±0.37	1.99±0.27	1.00±0.04	1.83±0.01	2.46±0.25
Plant defense										
BoDEF	1.00±0.07	2.24±0.70	6.62±0.69	1.00±0.44	3.11±1.22	9.27±6.66	3.96±0.22	1.00±0.08	2.21±0.01	9.59±0.19
BoPR	1.00±0.67	16.75±6.61	0.46±0.13	1.00±0.46	4.24±2.17	1.66±0.35	0.42±0.07	1.00±0.07	0.70±0.01	2.75±0.16

Supplementary Table S2.2. Continued.

MeJA (days after treatment)	Mature plants			
	Florets			
	0	1	2	4
18S rRNA	1.00±0.19	0.80±0.30	1.72±0.32	0.48±0.24
Glucosinolate biosynthesis				
BoCYP79B2	1.00±0.23	12.59±0.06	9.03±1.55	6.85±1.40
BoCYP83B1	1.00±0.04	4.39±0.24	3.15±0.04	3.65±0.56
BoCYP79F1	1.00±0.16	0.53±0.16	0.30±0.01	0.84±0.11
BoCYP83A1	1.00±0.07	5.47±0.78	4.93±1.28	2.43±0.95
Glucosinolate hydrolysis				
BoMYO	1.00±0.21	1.58±0.40	1.33±0.18	2.80±0.24
BoESP	1.00±0.00	1.12±0.07	1.10±0.15	1.32±0.17
Plant defense				
BoDEF	1.00±0.12	12.02±5.42	18.77±8.21	1.82±0.62
BoPR	1.00±0.67	5.20±1.34	14.67±0.31	1.51±0.97

Supplementary Table S2.3. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Pirate' broccoli seedlings with basal leaf wounding at 0 (control), 1 , 2 and 4 days after treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Apical leaves	Control	1.59±0.25 ab [y]	1.21±0.09 c	3.07±0.14 c	1.16±0.24 a	0.35±0.05 b	0.27±0.06 bc	1.03±0.08 c	0.16±0.02 b
	1 day after basal leaf wounding	1.93±0.05 ab	1.11±0.05 c	3.21±0.11 c	0.34±0.02 b	0.22±0.02 c	0.18±0.02 c	0.94±0.06 c	0.16±0.02 b
	2 days after basal leaf wounding	1.28±0.02 b	7.66±0.38 a	9.52±0.45 a	0.51±0.05 ab	0.22±0.03 c	0.58±0.06 ab	5.36±0.28 a	2.31±0.22 a
	4 days after basal leaf wounding	2.23±0.25 a	3.96±0.20 b	7.00±0.36 b	1.17±0.28 a	0.95±0.03 a	0.81±0.19 a	3.35±0.20 b	0.59±0.06 b
Basal leaves	Control	4.32±0.05 a	5.32±0.30 b	10.61±0.36 b	3.10±0.06 a	0.68±0.04 a	0.97±0.09 ab	4.19±0.22 b	1.13±0.08 b
	1 day after basal leaf wounding	3.31±0.21 ab	5.22±0.47 b	9.34±0.72 b	1.21±0.02 b	0.48±0.07 b	0.82±0.07 b	3.98±0.37 b	1.24±0.11 b
	2 days after basal leaf wounding	2.22±0.27 b	21.02±2.37 a	24.84±2.54 a	1.17±0.10 b	0.24±0.02 c	1.60±0.15 a	10.67±1.27 a	10.34±1.31 a
	4 days after basal leaf wounding	2.90±0.70 ab	8.30±2.72 b	12.36±3.70 b	2.17±0.60 ab	0.55±0.07 ab	1.16±0.28 ab	6.14±2.13 ab	2.15±0.59 b
Petioles	Control	2.09±0.08 a	0.54±0.05 a	3.72±0.20 a	1.25±0.07 a	0.70±0.05 a	1.09±0.10 a	0.25±0.04 a	0.29±0.02 a
	1 day after basal leaf wounding	1.20±0.20 b	0.56±0.02 a	2.38±0.29 b	0.68±0.10 b	0.46±0.10 ab	0.62 ±0.13 b	0.33±0.01 a	0.23±0.01 a
	2 days after basal leaf wounding	1.35±0.19 b	0.54±0.03 a	2.56±0.33 b	0.86 ±0.13 b	0.42±0.06 b	0.67±0.13 b	0.26±0.01 a	0.29±0.03 a
	4 days after basal leaf wounding	1.31±0.10 b	0.64±0.06 a	2.80±0.11 ab	0.82±0.07 b	0.44±0.06 ab	0.86±0.08 ab	0.32±0.03 a	0.32±0.04 a
Stems	Control [y]	2.82±0.14 a	0.25±0.04 b	4.03±0.08 a	1.90±0.15 ab	0.71±0.17 a	0.96±0.13 a	0.09±0.02 b	0.16±0.02 b
	1 day after basal leaf wounding	3.02±0.39 a	0.46±0.10 ab	4.46±0.41 a	2.22±0.13 a	0.48±0.11 a	0.97±0.05 a	0.18±0.04 ab	0.28±0.06 ab
	2 days after basal leaf wounding	3.07±0.26 a	0.59±0.04 a	4.63±0.28 a	2.25±0.14 a	0.56±0.07 a	0.98±0.04 a	0.19±0.02 a	0.40±0.02 a
	4 days after basal leaf wounding	2.24±0.05 a	0.60±0.07 a	3.69±0.19 a	1.72±0.04 b	0.46±0.02 a	0.85±0.08 a	0.18±0.02 ab	0.41±0.05 a

[x] 4MSOB: 4-methylsulphinylbutyl GS, 3IM: 3-indolymethyl GS, N-3IM: N-methoxy-3-indolylmethyl GS. The total aliphatic and indolyl GS concentrations include the concentration of 3-methylsulphinylpropyl GS and (2R) 2-hydroxy-3-butenyl GS, and 4-methoxy-3-indolylmethyl GS, respectively.

[y] Means within each columm and tissue type with different letters are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

Supplementary Table S2.4. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Pirate' broccoli seedlings at 0 (control), 1, 2 and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Apical leaves	Control [y]	1.59±0.25 a [z]	1.21±0.09 b	3.07±0.14 b	1.16±0.24 a	0.35±0.05 a	0.27±0.06 a	1.03±0.08 c	0.16±0.02 b
	1 day after MeJA treated	1.04±0.01 a	6.52±0.25 a	8.05±0.25 a	0.63±0.04 a	0.18±0.01 bc	0.49±0.02 a	3.98±0.13 a	2.55±0.15 a
	2 days after MeJA treated	1.23±0.01 a	6.24±0.12 a	7.89±0.28 a	0.76±0.09 a	0.25±0.02 ab	0.41±0.13 a	4.40±0.06 a	1.84±0.15 ab
	4 days after MeJA treated	1.37±0.17 a	5.12±1.79 ab	7.07±2.11 ab	0.83±0.15 a	0.11±0.03 c	0.58±0.19 a	2.41±0.64 b	2.71±1.15 a
Basal leaves	Control	4.32±0.05 a	5.32±0.30 b	10.61±0.36 b	3.10±0.06 a	0.68±0.04 a	0.97±0.09 b	4.19±0.22 b	1.13±0.08 c
	1 day after MeJA treated	2.99±0.89 a	15.16±1.08 a	19.40±2.25 a	2.58±0.82 a	0.19±0.06 b	1.25±0.29 ab	5.15±0.58 b	10.00±0.51 a
	2 days after MeJA treated	3.22±0.26 a	17.39±1.06 a	22.27±1.28 a	2.67±0.27 a	0.22±0.01 b	1.66±0.04 a	8.97±0.43 a	8.42±0.86 ab
	4 days after MeJA treated	3.34±0.26 a	15.03±1.64 a	19.88±1.97 a	2.78±0.28 a	0.19±0.03 b	1.51±0.19 ab	7.78±0.81 a	7.23±0.88 b
Petioles	Control	2.09±0.08 a	0.54±0.05 b	3.72±0.20 a	1.25±0.07 a	0.70±0.05 a	1.09±0.10 a	0.25±0.04 b	0.29±0.02 b
	1 day after MeJA treated	2.22±0.41 a	1.69±0.26 a	4.94±0.80 a	1.71±0.29 a	0.39±0.12 ab	1.02±0.17 a	0.33±0.07 b	1.36±0.22 a
	2 days after MeJA treated	1.63±0.18 a	1.61±0.14 a	4.23±0.24 a	1.19±0.17 a	0.31±0.04 b	0.99±0.19 a	0.57±0.06 a	1.04±0.09 a
	4 days after MeJA treated	1.97±0.60 a	1.75±0.24 a	4.82±0.94 a	1.69±0.49 a	0.18±0.10 b	1.10±0.23 a	0.38±0.00 b	1.37±0.24 a
Stems	Control [y]	2.82±0.14 a	0.25±0.04 b	4.03±0.08 b	1.90±0.15 ab	0.71±0.17 a	0.96±0.13 a	0.09±0.02 b	0.16±0.02 b
	1 day after MeJA treated	2.34±0.10 a	3.29±0.35 a	6.31±0.48 a	1.95±0.04 ab	0.29±0.07 a	0.68±0.05 a	0.23±0.02 a	3.06±0.34 a
	2 days after MeJA treated	2.29±0.40 a	2.54±0.13 a	5.76±0.58 ab	1.60±0.35 b	0.47±0.08 a	0.94±0.07 a	0.31±0.03 a	2.23±0.10 a
	4 days after MeJA treated	3.02±0.25 a	2.56±0.36 a	6.71±0.69 a	2.43±0.19 a	0.52±0.09 a	1.13±0.24 a	0.26±0.06 a	2.30±0.31 a

([x and y]: see table S2.3)

Supplementary Table S2.5. Changes in GS concentrations (± SE; μM/g DW) in different tissues of mature 'Pirate' broccoli plants 0 (control), 1, 2, and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Florets	Control	7.77±1.17 a [y]	4.81±1.75 c	13.47±2.91 c	2.52±0.19 a	4.97±1.26 a	0.89±0.15 c	3.58±1.39 b	1.20±0.38 c
	1 day after MeJA treated	10.03±0.85 a	15.12±1.50 ab	27.00±1.64 ab	2.94±0.25 a	6.63±0.69 a	1.85±0.11 b	7.14±0.40 a	7.95±1.10 b
	2 day after MeJA treated	9.64±0.07 a	12.91±3.09 b	24.47±3.31 b	2.43±0.15 a	6.89±0.13 a	1.91±0.19 b	4.73±0.82 ab	8.06±2.33 b
	4 day after MeJA treated	8.58±0.40 a	22.93±1.68 a	34.70±1.98 a	3.23±0.31 a	5.02±0.27 a	3.19±0.26 a	6.20±0.63 ab	16.68±1.04 a
Apical leaves	Control	2.72±0.17 b	3.01±0.15 b	6.61±0.36 b	1.60±0.10 b	1.05±0.09 a	0.88±0.09 b	2.39±0.12 b	0.63±0.06 ab
	1 day after MeJA treated	2.40±0.33 b	3.29±0.61 ab	6.78±1.08 b	1.93±0.24 ab	0.33±0.09 c	1.08±0.21 b	2.63±0.45 ab	0.64±0.16 ab
	2 day after MeJA treated	2.40±0.18 b	3.52±0.28 ab	7.09±0.53 b	1.81±0.17 b	0.44±0.07 bc	1.17±0.13 ab	2.90±0.23 ab	0.59±0.06 b
	4 day after MeJA treated	3.74±0.46 a	4.43±0.55 a	9.76±0.57 a	2.57±0.31 a	0.74±0.18 ab	1.59±0.11 a	3.49±0.48 a	0.92±0.10 a
Basal leaves	Control	1.29±0.13 b	0.37±0.07 b	1.85±0.22 b	0.37±0.05 b	0.76±0.09 b	0.19±0.03 b	0.20±0.04 bc	0.17±0.03 b
	1 day after MeJA treated	2.47±0.65 a	0.34±0.08 b	3.41±0.59 a	0.73±0.23 a	1.23±0.28 a	0.61±0.05 a	0.14±0.05 c	0.20±0.04 ab
	2 day after MeJA treated	1.19±0.02 b	0.69±0.04 a	2.15±0.09 b	0.32±0.04 b	0.81±0.03 ab	0.27±0.03 b	0.42±0.02 a	0.27±0.03 ab
	4 day after MeJA treated	0.96±0.06 b	0.66±0.15 a	1.85±0.21 b	0.24±0.02 b	0.66±0.04 b	0.23±0.04 b	0.31±0.05 ab	0.34±0.09 a
Stems	Control [y]	2.45±0.14 a	0.27±0.02 b	3.24±0.14 a	1.18±0.15 a	1.13±0.09 a	0.52±0.03 a	0.08±0.01 b	0.19±0.01 bc
	1 day after MeJA treated	2.52±0.79 a	0.28±0.05 b	3.41±0.90 a	0.88±0.25 a	1.30±0.41 a	0.62±0.09 a	0.10±0.02 ab	0.19±0.04 c
	2 day after MeJA treated	3.51±0.23 a	0.38±0.01 a	4.47±0.23 a	1.07±0.09 a	1.87±0.11 a	0.58±0.02 a	0.12±0.00 a	0.26±0.01 b
	4 day after MeJA treated	2.32±0.26 a	0.44±0.03 a	3.37±0.35 a	0.84±0.04 a	1.17±0.31 a	0.61±0.10 a	0.10±0.00 ab	0.34±0.03 a

([x and y]: see table S2.3)

Supplementary Table S2.6. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Green Magic' broccoli seedlings at 0 (control), 1, 2, and 4 days after basal leaf wounding.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Apical leaves	Control	0.59±0.03 a [y]	0.66±0.02 b	1.63±0.02 b	0.20±0.00 b	0.22±0.02 a	0.38±0.03 a	0.45±0.02 b	0.20±0.03 b
	1 day after basal leaf wounding	0.56±0.07 a	0.67±0.07 b	1.40±0.15 b	0.32±0.05 ab	0.00±0.00 b	0.18±0.02 b	0.47±0.04 b	0.19±0.03 b
	2 days after basal leaf wounding	0.80±0.25 a	0.72±0.02 b	1.74±0.26 b	0.51±0.17 ab	0.00±0.00 b	0.21±0.03 b	0.52±0.03 b	0.20±0.01 b
	4 days after basal leaf wounding	1.06±0.11 a	1.22±0.13 a	2.42±0.18 a	0.68±0.09 a	0.03±0.03 b	0.14±0.01 b	0.67±0.06 a	0.55±0.08 a
Basal leaves	Control	2.80±0.70 a	3.17±0.91 a	7.07±1.78 a	1.46±0.36 b	0.56±0.12 a	1.11±0.19 a	1.58±0.33 a	1.57±0.58 b
	1 day after basal leaf wounding	3.54±0.69 a	4.76±0.36 a	9.35±0.30 a	2.53±0.50 ab	0.00±0.00 b	1.05±0.06 a	1.91±0.14 a	2.83±0.22 ab
	2 days after basal leaf wounding	4.21±0.06 a	6.33±0.51 a	12.12±0.74 a	2.97±0.02 ab	0.00±0.00 b	1.59±0.28 a	2.07±0.22 a	4.25±0.32 ab
	4 days after basal leaf wounding	5.37±1.02 a	6.61±1.58 a	13.32±2.95 a	3.81±0.73 a	0.00±0.00 b	1.33±0.38 a	1.62±0.21 a	5.00±1.38 a
Petioles	Control	2.16±0.15 b	0.60±0.08 ab	3.35±0.18 b	1.29±0.13 b	0.36±0.01 b	0.59±0.07 a	0.12±0.02 a	0.48±0.07 ab
	1 day after basal leaf wounding	2.65±0.63 b	0.41±0.06 b	3.56±0.65 b	1.70±0.44 ab	0.30±0.03 b	0.51±0.03 a	0.11±0.02 a	0.30±0.04 c
	2 days after basal leaf wounding	2.98±0.41 ab	0.52±0.04 b	4.36±0.40 ab	1.92±0.32 ab	0.40±0.02 ab	0.86±0.21 a	0.15±0.03 a	0.37±0.02 bc
	4 days after basal leaf wounding	4.54±0.61 a	0.81±0.03 a	6.19±0.64 a	2.90±0.45 a	0.51±0.06 a	0.84±0.05 a	0.18±0.01 a	0.63±0.04 a
Stems	Control [y]	3.14±0.27 b	0.55±0.05 ab	4.44±0.20 b	1.99±0.18 c	0.56±0.06 a	0.75±0.10 ab	0.15±0.02 a	0.40±0.04 ab
	1 day after basal leaf wounding	3.77±0.40 b	0.35±0.03 b	4.79±0.50 b	2.61±0.31 bc	0.41±0.04 a	0.66±0.08 b	0.11±0.01 a	0.24±0.03 b
	2 days after basal leaf wounding	4.35±0.47 b	0.45±0.05 ab	5.88±0.41 b	2.85±0.24 b	0.68±0.13 a	1.08±0.11 ab	0.14±0.02 a	0.31±0.03 b
	4 days after basal leaf wounding	6.00±0.12 a	0.71±0.13 a	7.80±0.35 a	4.21±0.12 a	0.63±0.09 a	1.09±0.15 a	0.15±0.03 a	0.56±0.10 a

([x and y]: see table S2.3)

Supplementary Table S2.7. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Green Magic' broccoli seedlings 0 (control), 1, 2 and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Apical leaves	Control	0.59±0.03 b[y]	0.66±0.02 b	1.63±0.02 b	0.20±0.00 b	0.22±0.02 a	0.38±0.03 a	0.45±0.02 b	0.20±0.03 b
	1 day after MeJA treated	1.19±0.26 a	5.39±0.27 a	6.93±0.33 a	0.63±0.18 a	0.23±0.02 a	0.35±0.00 a	0.59±0.06 b	4.78±0.33 a
	2 days after MeJA treated	0.73±0.12 ab	6.17±0.94 a	7.24±1.07 a	0.38±0.08 ab	0.09±0.04 b	0.34±0.01 a	1.12±0.06 a	5.04±0.89 a
	4 days after MeJA treated	0.74±0.06 ab	7.58±1.77 a	8.68±1.85 a	0.46±0.05 ab	0.08±0.02 b	0.36±0.05 a	0.42±0.07 b	7.16±1.71 a
Basal leaves	Control	2.80±0.70 a	3.17±0.91 b	7.07±1.78 b	1.46±0.36 a	0.56±0.12 a	1.11±0.19 a	1.58±0.33 a	1.57±0.58 c
	1 day after MeJA treated	3.28±0.86 a	9.05±2.51 ab	13.42±3.24 ab	2.21±0.62 a	0.06±0.05 b	1.09±0.15 a	0.74±0.22 b	8.29±2.30 b
	2 days after MeJA treated	2.05±0.19 a	15.83±1.06 a	19.34±1.27 a	1.25±0.11 a	0.08±0.07 b	1.46±0.09 a	0.96±0.05 ab	14.84±1.01 a
	4 days after MeJA treated	2.33±0.37 a	9.84±2.39 ab	13.30±2.94 ab	1.67±0.21 a	0.09±0.02 b	1.13±0.19 a	0.42±0.10 b	9.40±2.28 ab
Petioles	Control	2.16±0.15 b	0.60±0.08 b	3.35±0.18 b	1.29±0.13 b	0.36±0.01 a	0.59±0.07 b	0.12±0.02 b	0.48±0.07 b
	1 day after MeJA treated	4.30±0.64 a	1.27±0.09 b	6.27±0.71 a	2.99±0.44 a	0.35±0.07 a	0.69±0.09 b	0.14±0.00 b	1.13±0.09 b
	2 days after MeJA treated	3.36±0.19 ab	2.20±0.30 a	6.49±0.29 a	2.30±0.15 ab	0.23±0.01 ab	0.93±0.16 ab	0.19±0.01 a	2.01±0.29 a
	4 days after MeJA treated	5.03±0.82 a	2.26±0.27 a	8.54±1.14 a	3.65±0.62 a	0.11±0.04 b	1.24±0.13 a	0.14±0.02 ab	2.12±0.25 a
Stems	Control [y]	3.14±0.27 b	0.55±0.05 b	4.44±0.20 b	1.99±0.18 b	0.56±0.06 a	0.75±0.10 b	0.15±0.02 ab	0.40±0.04 b
	1 day after MeJA treated	4.09±0.61 ab	2.25±0.16 a	7.20±0.49 a	2.81±0.43 ab	0.19±0.05 b	0.87±0.03 b	0.10±0.01 b	2.15±0.16 a
	2 days after MeJA treated	4.30±0.57 ab	3.00±0.45 a	8.58±1.08 a	2.90±0.35 ab	0.32±0.01 b	1.28±0.08 a	0.16±0.01 a	2.84±0.44 a
	4 days after MeJA treated	5.07±0.15 a	2.12±0.36 a	8.43±0.53 a	3.56±0.04 a	0.19±0.06 b	1.24±0.13 a	0.11±0.01 b	2.02±0.35 a

([x and y]: see table S2.3)

Supplementary Table S2.8. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Green Magic' mature broccoli plants 0 (control), 1, 2, and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Florets	Control	7.01±1.36 a [y]	2.21±0.25 b	10.35±1.96 c	2.76±0.23 a	3.07±0.86 a	1.14±0.37 b	0.81±0.18 a	1.38±0.38 b
	1 day after MeJA treated	8.37±0.63 a	4.06±0.69 b	13.92±1.16 bc	2.81±0.23 a	3.38±0.20 a	1.49±0.10 b	0.92±0.06 a	3.12±0.64 b
	2 day after MeJA treated	10.82±1.06 a	9.94±1.89 a	23.68±2.83 ab	4.30±0.43 a	3.58±0.54 a	2.92±0.28 a	1.26±0.16 a	8.66±1.72 a
	4 day after MeJA treated	14.46±3.93 a	8.58±0.90 a	26.26±4.75 a	5.78±1.75 a	5.18±1.34 a	3.22±0.43 a	1.13±0.23 a	7.33±0.70 a
Apical leaves	Control	1.99±0.18 b	2.30±0.04 b	5.21±0.33 b	1.33±0.10 b	0.29±0.08 b	0.92±0.12 b	0.23±0.09 b	2.00±0.06 b
	1 day after MeJA treated	5.40±0.48 ab	5.42±0.53 a	12.78±0.31 ab	2.81±0.31 ab	1.42±0.20 a	1.96±0.11 ab	0.76±0.11 a	4.57±0.57 ab
	2 day after MeJA treated	7.29±1.67 a	6.68±0.84 a	16.37±2.52 a	4.31±1.12 a	1.29±0.20 a	2.41±0.26 a	0.47±0.08 ab	6.13±0.84 a
	4 day after MeJA treated	6.00±1.35.ab	5.86±1.18 a	14.57±3.14 a	3.92±0.83 ab	0.86±0.25 ab	2.71±0.62 a	0.35±0.10 b	5.43±1.10 a
Basal leaves	Control	0.60±0.20 a	0.49±0.10 c	1.27±0.28 c	0.30±0.12 b	0.10±0.05 a	0.18±0.11 b	0.15±0.09 a	0.32±0.02 c
	1 day after MeJA treated	1.22±0.12 a	1.94±0.34 a	3.71±0.42 a	0.56±0.03 ab	0.32±0.09 a	0.56±0.09 a	0.31±0.03 a	1.61±0.35 a
	2 day after MeJA treated	1.08±0.34 a	0.95±0.09 bc	2.25±0.31 bc	0.47±0.17 ab	0.22±0.08 a	0.22±0.09 b	0.29±0.01 a	0.64±0.09 bc
	4 day after MeJA treated	1.44±0.31 a	1.51±0.23 ab	3.56±0.41 ab	0.85±0.18 a	0.13±0.06 a	0.60±0.05 a	0.14±0.02 a	1.34±0.22 ab
Stems	Control [y]	2.12±0.42 b	0.15±0.01 a	2.98±0.27 b	1.27±0.27 b	0.46±0.09 c	0.71±0.16 a	0.03±0.00 b	0.12±0.01 a
	1 day after MeJA treated	4.03±0.25 ab	0.23±0.03 a	4.58±0.32 ab	1.80±0.25 ab	1.18±0.04 bc	0.32±0.04 b	0.05±0.00 b	0.17±0.03 a
	2 day after MeJA treated	5.89±0.94 a	0.37±0.10 a	6.72±1.13 a	2.27±0.18 a	2.38±0.46 a	0.46±0.09 ab	0.13±0.04 a	0.24±0.07 a
	4 day after MeJA treated	4.56±0.48 a	0.35±0.07 a	5.45±0.50 a	2.21±0.34 ab	1.55±0.09 ab	0.54±0.03 ab	0.08±0.02 ab	0.28±0.06 a

([x and y]: see table S2.3)

Supplementary Table S2.9. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'VI-158' broccoli seedlings 0 (control), 2, and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Leaves	Control	4.09±0.55a [y]	2.13±0.17b	6.60±0.36b	1.88±0.46a	1.85±0.51a	0.38±0.11a	1.55±0.22b	0.45±0.05a
	2 days after MeJA treated	2.11±0.32b	16.25±1.66a	19.37±2.13a	0.81±0.24a	1.10±0.44a	1.01±0.28a	11.58±3.36a	4.57±1.72a
	4 days after MeJA treated	2.16±0.26b	14.62±1.15a	17.67±1.09a	1.15±0.19a	0.81±0.07a	0.89±0.12a	13.31±0.92a	1.25±0.54a
Petioles	Control	3.00±0.19a	2.19±0.16a	6.79±0.29a	1.60±0.05a	1.07±0.12a	1.60±0.19a	0.64±0.16b	1.52±0.02a
	2 days after MeJA treated	1.98±0.26a	3.40±0.40a	6.98±0.74a	0.86±0.24b	0.89±0.06ab	1.60±0.26a	1.48±0.21ab	1.90±0.49a
	4 days after MeJA treated	2.51±0.34a	3.74±0.64a	7.76±1.24a	1.61±0.20a	0.47±0.17b	1.51±0.31a	2.12±0.38a	1.57±0.27a
Stems	Control	3.96±0.20a	1.28±0.09a	6.53±0.22a	2.01±0.09a	1.61±0.21a	1.29±0.03a	0.39±0.03a	0.86±0.11a
	2 days after MeJA treated	4.27±0.88a	6.78±3.04a	13.22±4.29a	1.91±0.49a	2.08±0.72a	2.17±0.45a	2.79±1.70a	3.95±1.35a
	4 days after MeJA treated	4.06±0.51a	2.64±0.64a	8.13±0.97a	2.61±0.36a	1.03±0.11a	1.43±0.29a	1.13±0.21a	1.49±0.47a
Roots	Control	1.11±0.30a	0.66±0.13a	6.21±1.20a	0.36±0.12a	0.53±0.13a	4.44±0.85a	0.08±0.04a	0.54±0.09a
	2 days after MeJA treated	0.97±0.31a	2.29±1.40a	7.06±2.27a	0.09±0.04a	0.70±0.24a	3.79±0.58a	0.49±0.34a	1.79±1.06a
	4 days after MeJA treated	1.41±0.27a	0.90±0.09a	6.62±1.15a	0.39±0.15a	0.84±0.10a	4.30±0.81a	0.10±0.01a	0.79±0.08a

([x and y]: see table S2.3)

Supplementary Table S2.10. Changes in GS concentrations (± SE; μM/g DW) in different tissues of 'Brigidier' broccoli seedlings 0 (control), 2, and 4 days after MeJA treatment.

Plant tissue	Treatment	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Leaves	Control	2.38±0.27a [y]	2.92±0.33c	6.12±0.60b	0.71±0.19a	1.63±0.28a	0.82 ±0.12a	2.12±0.21c	0.72±0.13b
	2 days after MeJA treated	2.92±0.55a	9.20±1.07b	13.19±1.38a	0.79±0.13a	1.95±0.48a	1.07 ±0.21a	4.70±0.35b	4.41±0.71a
	4 days after MeJA treated	1.67±0.22a	12.88±1.02a	15.81±1.41a	0.48±0.14a	1.06±0.07a	1.25±0.22a	6.97±0.41a	5.72±1.36a
Petioles	Control	4.36±0.54a	2.06±0.22a	7.87±0.46a	1.56±0.37a	2.68±0.16a	1.45±0.08a	0.81±0.07c	1.05±0.13b
	2 days after MeJA treated	3.51±0.25ab	2.16±0.26a	7.37±0.60a	1.07±0.09a	2.37±0.15a	1.70±0.11a	0.58±0.07b	1.41±0.19a
	4 days after MeJA treated	2.91±0.19b	7.02±2.15a	12.61±2.55a	0.79±0.18a	1.98±0.19a	2.68±0.38a	2.03±0.69a	4.82±1.47a
Stems	Control	2.70±0.16a	0.63±0.12a	3.89±0.27a	0.74±0.11a	1.89±0.22a	0.56±0.03b	0.24±0.05a	0.36±0.09b
	2 days after MeJA treated	2.50±0.20a	1.78±0.05a	5.49±0.17a	0.79±0.16a	1.63±0.05ab	1.21±0.07b	0.34±0.01a	1.34±0.01ab
	4 days after MeJA treated	2.68±0.14a	3.53±1.60a	7.70±2.07a	1.06±0.07a	1.51±0.13b	1.49±0.38a	0.58±0.24a	2.89±1.33a
Roots	Control	0.68±0.07a	0.51±0.02a	3.76±0.22a	0.22±0.05ab	0.45±0.05a	2.57±0.14a	0.05±0.01a	0.46±0.01a
	2 days after MeJA treated	1.14±0.11a	0.79±0.09a	5.31±0.39a	0.10±0.01b	1.01±0.11a	3.38±0.21a	0.18±0.03a	0.61±0.06a
	4 days after MeJA treated	1.29±0.34a	1.42±0.53a	7.54±1.96a	0.32±0.05a	0.96±0.30a	4.83 ±1.08a	0.23±0.11a	1.19±0.43a

([x and y]: see table S2.3)

CHAPTER 3

Metabolic profiling of methyl jasmonate-mediated changes in broccoli (*Brassica oleracea* L. ssp *Italica*) tissues

3.1. Abstract

Methyl jasmonate (MeJA) is an endogenous plant elicitor associated with up-regulation of the biosynthesis of several health-promoting phytochemical compounds such as glucosinolates, phenolics and ascorbic acid. Consumption of broccoli with increased levels of these compounds has been associated with reduced risk of cancer and other degenerative diseases. Metabolic profiling of hydrophilic metabolites using gas chromatography-mass spectrometry (GC-MS) demonstrated accumulation of various phenolics, ascorbates and amino acids in different broccoli tissues after MeJA treatment. Distinct changes in carbohydrate levels were also observed between different tissues of seedlings and floret tissues of broccoli plants at harvest maturity after treatment. Hexose sugars were significantly decreased in vegetative tissues of broccoli seedlings, whereas the florets of market maturity broccoli exhibited increased carbohydrates after MeJA treatment. These differences suggest that the carbon metabolism is differentially modulated by MeJA treatment in different tissue types depending on sink-source relationships. Reduced levels of hexose sugars and tricarboxylic acid (TCA) intermediates after MeJA treatment may reflect the increased requirement for carbon and energy needed to drive secondary product biosynthesis to accumulate defense metabolites against insects and other herbivores.

3.2. Introduction

Jasmonic acid (JA) and methyl jasmonate (MeJA), a volatile methylester of jasmonic acid are endogeneous plant signaling molecules that modulate the expression of genes related

with the plant's defense system in response to stress conditions (Pauwels et al., 2008). Recent transcript and metabolite profiling studies have demonstrated that JAs stimulate expression of genes involved in glucosinolate, phenylpropanoid, flavonoid, monolignol and ascorbic acid metabolism, leading to the accumulation of these metabolites in plant tissues (Broeckling et al., 2005; Sasaki-Sekimoto et al., 2005; Farag et al., 2008; reviewed in Pauwels et al., 2009). Application of exogeneous JAs influenced export and distribution of photosynthates in *Populus*, suggesting that JAs may be associated with metabolic partitioning of carbon resources to allocate energy to biochemical defense mechanisms to protect vulnerable plant tissues from biotic and abiotic stress (Babst et al., 2004; Ferrieri et al., 2005).

Among the various metabolites, indolyl glucosinolates dramatically respond to MeJA signaling in *Brassica* species (Bodnaryk, 1994; Doughty et al., 1995; Kliebenstein et al., 2002; van Dam et al., 2003). Sink tissues (apical leaves of seedlings and florets of mature plants) accumulate indolyl glucosinolates with MeJA treatments (Chapter 2). This organ-specific accumulation of indolyl glucosinolates in broccoli suggests that MeJA may also modulate changes in primary metabolism to provide substrate and energy for the generation of defense substances. Increases in indolyl glucosinolates may be accompanied by the changes in their precursor amino acid levels (Van Dam and Oomen, 2008). Metabolic profiling in *Medicago trucatula* (Broeckling et al., 2005), *Arabidopsis* (Hendrawati et al., 2006), and *Brassica rapa* (Liang et al., 2006) has demonstrated that application of exogeneous MeJA mediates changes in levels of carbohydrates, organic acids and amino acids. Metabolic profiling was also employed to analyze biochemical changes in aspen leaves undergoing transition from a sink tissue to a source of photosynthates (Jeong et al., 2004).

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is a dietary source of several health-promoting phytochemicals including glucosinolates, carotenoids, tocopherols, and ascorbic acid (Kushad et al., 1999; Kurilich et al., 1999). The health-promoting properties of broccoli has increased the popularity of this vegetable and provided value-added economic benefits for its commercial production. In spite of the nutritional importance of broccoli, information on overall primary metabolism associated with the biosynthesis and accumulation of health-promoting phytochemical compounds is incomplete.

The aim of this study was to analyze metabolic changes mediated by exogeneous MeJA treatment of broccoli. Biochemical feactures observed between seedling vegetative and floret reproductive tissues after MeJA treatment were analyzed to elucidate the influences of MeJA on metabolism and nutritional quality of broccoli in an organ-specific manner.

3.3. Materials and methods

3.3.1. Plant material

Seed of the broccoli cultivars 'Green Magic' (Sakata Seed Co.) was germinated in small pots filled with sunshine® LC1 professional soil mix and transplanted into 5 cm pots and grown for six weeks in a greenhouse at the University of Illinois at Champaign-Urbana under a 25℃/15℃ and 14-h/10-h day/night temperature regime and with supplemental light. For mature plants, additional seedlings were transplanted into 22.8 cm pots and grown under the same greenhouse conditions until heads reach harvest maturity. After transplanting, plants were fertilized with a solution of 20N-20P-20K twice a week. Experimental design was a randomized complete block with three replicates of 3 plants spaced 30 cm apart with 30 cm between rows on greenhouse benches.

3.3.2. MeJA treatment

Six-week old broccoli seedlings and mature broccoli plants were subjected to MeJA treatments. All aerial plant parts were sprayed with 250 µM MeJA (Sigma-aldrich, St. Louis, MO) containing 0.1% Triton X-100 (Sigma-aldrich, St. Louis, MO) until plant surfaces were fully saturated with MeJA solution. Control plants were sprayed with water containing 0.1% Triton X-100. Plants were spray treated in isolation and completely dried prior to being returned to greenhouse benches.

Broccoli seedling and mature plant tissue samples were harvested 4 days after treatment. The duration between MeJA treatment and sample harvest time was based on preliminary glucosinolate profiling data which showed the maximum levels of glucosinolates 4 days after treatment. In six week old seedlings, apical (second and third leaves including emerging shoots from the apical meristem) and basal (4th-6th leaf node) leaves were harvested. In mature broccoli plants, immature florets were harvested at fresh market maturity. The three plant subsamples were combined to generate each of the three replicates for control and treated samples. The samples were frozen in liquid nitrogen, lyophilized, ground into powder, and stored at -20℃ until GC-MS analysis.

3.3.3. Metabolite profiling using GC-MS

Control and treated samples of apical and basal leaves of six week old broccoli seedlings, and florets of mature broccoli plants were subjected to GC-MS metabolite profiling. To analyze hydrophilic metabolites, 15 mg aliquots of lyophilized tissues were extracted with 80% (v / v) aqueous methanol, and the separated polar phases were evaporated and derivatized with MSTFA as described by Fiehn et al. (2000) and Roessner et al. (2000) with minor modification: 90 min at 50 °C with 80 µl of of methoxyamine hydrochloride in pyridine (20 mg ml^{-1}) with following 60

min treatment at 50^0C with 80 µl MSTFA. A 10 µl of an internal standard (C31 fatty acid) was added prior to trimethylsilylation. GC with quadrupole mass spectrometric detection was performed using an Agilent 7890A gas chromatograph, an Agilent 5975C mass selective detector, and an Agilent 7683B autosampler (Agilent Inc., Palo Alto, CA) in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Derivartized extract (1 µl) was injected with a split ratio of 5:1 onto a 60 m × 0.25 mm (i.d.), 0.25 µm film HP-5MS column (Agilent Inc.) with the injection temperature at 250 ℃, the interface set to 250 ℃, and the ion source adjusted to 230 ℃. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperature program was 5 min isothermal heating at 70 ℃, followed by oven temperature increase of 5 ℃ /min to 310 ℃ and a final 20 min at 310 ℃. Mass spectra were recorded with a scan range of 25 to 800 m/z. The spectra of all chromatogram peaks were evaluated as described by Lozovaya et al. (2006). To calculate relative abundance of metabolites, peak areas were divided by peak area of the internal standard, hentriacontanoic acid (50 µg/mL), and normalized on the basis of sample dry weight. All data were $\log_{10}$ transformed for statistical analysis.

3.3.4. Statistical analysis

Relative abundance of metabolites in different samples were analyzed by t-test, one-way analysis of variance (ANOVA) and Fisher's LSD tests with P <0.05 using the SAS statistical analysis package (SAS version 8.0). Principal component analysis (PCA) and hierarchical clustering (HCA) were performed using SIMCA-P+ (12.0.0.0) program (Umetrics AB, Tvistevdgen 48 Umea 907 19, Sweden) and MetaboAnalyst, an online web server for metabolomics data analysis (http://www.metaboanalyst.ca), respectively. PCA was used to calculate the loadings of specific metabolites for different tissue and treatment sample clustering.

HCA was also used to separate different samples into clusters using a variety of dissimilarity measures (Xia et al., 2009). The result of HCA was presented as a dendrogram and heat map.

3.4. Results

3.4.1. Metabolic features observed in different broccoli tissue types

Polar metabolites from methanol extracts were profiled using GC-MS to analyze changes in relative abundance between different samples. According to the position of leaf internodes in six week old broccoli seedlings, apical and basal leaves were considered as the actively growing young (sink tissue) and mature (source tissue) leaf tissues, respectively. Due to minor differences in ratio of leaf expansion and limited number of leaves in seedlings, leaf plastochron index analysis was not applied in our experiment. Floret tissue of mature broccoli plants was considered a sink organ, which is the consumed edible portion of broccoli.

A total of ninety different metabolites were identified with a high levels of certainty in apical and basal leaf tissues of broccoli seedlings, and florets of mature plants with or without MeJA treatment (Supplementary Table S3.1). HCA and PCA analysis were employed to analyze the pattern of association among all metabolites. HCA analysis identified clearly separated clusters depending on tissue type and treatment (Figure 3.1). Apical and basal leaf tissues formed distinct metabolite clusters which were more closely related to each other than to the distantly clustered floret tissues. PCA analysis validated these clustering patterns (Figure 3.2). Seedling vegetative tissues (apical and basal leaves) and florets separated primarly on the first component, which explained 59% of the variation primarly contributed by different tissue types, with control and MeJA treatment forming distinct clusters based on the second component, which explained 15% of overall metabolite variance (Figure 3.2). These results indicate that the metabolite

profiles displayed sufficient variation to be distinguished between different tissue types and between control and MeJA treatment.

Overall trends in relative abundance of metabolites in individual samples of different tissue types and treatments were presented by a heat map (Figure 3.1). The metabolites clustered by HCA analysis (top horizontal axis) displayed similar patterns of abundance but varied between different tissue types and MeJA treatment (Figure 3.1). ANOVA was performed to analyze significant differences in relative abundances (represented by mean normalized peak area) of individual metabolites between different tissue types with or without MeJA treatment. Of the 90 metabolites identified, 60 and 71 metabolites showed significant differences at $P < 0.05$ among the different tissue types with control and MeJA treatment, respectively. These metabolites were grouped according to the rank relative abundances displayed between apical and basal leaves of seedlings, and between florets of mature plants and apical and basal leaves of seedlings (Table 3.1). Compared to basal leaves of seedlings, apical leaf tissue showed relatively higher levels of most of sugars, sugar alcohols, organic acids, phenolics and amino acids. The level of hexose and hexose phosphates, fructose, glucose, glucose-6-P, galactose, mannose and mannose-6-P were significantly higher in apical leaves of seedlings (AL>BL), whereas basal leaves showed relatively higher levels of pentose (xylulose and ribose), heptose (sedoheptulose), and trisaccharide (raffinose) (BL>AL). Basal leaves contained 70% and 72% greater amounts of gulose and raffinose, respectively. The tricarboxylic acid (TCA) cycle intermediates, malic acid, succinic acid, fumaric acid, citric acid, and aconitic acid, and phosphorespiratory substrate glycolic acid showed overall higher concentrations in apical leaves of seedlings compared to basal leaves (Supplementary Table S3.1), but only citric acid and aconitic acid were significantly different between two tissue types at $P < 0.05$ (Table 3.1). Higher levels of TCA cycle

intermediates, hexose sugars, and amino acids in apical leaves of seedlings indicates photosynthetic development and sink to source transition. This trend was similar to the metabolic features observed between expanding and mature leaves of aspen (*Populus tremuloides* Michx.) (Jeong et al., 2004).

Florets of mature broccoli plants contained abundant levels of amino acids and polyols (sugar alcohols) except glycerol compared to the apical and basal leaf tissues of broccoli seedlings. Gulose, erythrose, maltose and xylulose were discriminative carbohydrates not detected in floret tissues of both control and MeJA treated plants under our protocol.

3.4.2. MeJA mediated changes in carbon metabolism in broccoli

Changes in the levels of individual metabolites mediated by MeJA treatment were analyzed in different tissue types of broccoli with or without MeJA treatment. In apical leaves of seedlings, disaccharides (sucrose, maltose and isomaltose) and trisaccharides (raffinose) levels were significantly increased, whereas the hexose sugars, fructose, galactopyranose, glulose, glucose-6-P and sorbose, and the TCA intermediates, malic acid, succinic acid, aconitic acid and fumaric acid levels decreased after MeJA treatment (Table 3.2). Accumulation of polyols were observed in all tissues tested in our experiment after MeJA treatment. Basal leaves of seedlings demonstrated significantly increased levels of pentose sugars including arabinose, xylose and xylulose, and di- and trisaccharides, maltose and raffinose with decreases in hexoses, galactose and glactopyronase, and the TCA intermediates, succinic acid and malic acid (Table 3.2). Decreasing levels of the hexoses and TCA cycle intermediates were observed in both apical and basal leaf of seedling after MeJA treatment. These trends may reflect enhanced demand for carbon by downstream biochemical pathways after MeJA treatment.

In the floret tissues, only mannose-6-P was significantly decreased, while most of carbohydrates exhibited increases after MeJA treatment (Table 3.2 and Supplementary Table S3.1). No significant reduction in TCA intermediates were observed in florets of mature plants after treatment. These results are distinct from the changes in apical and basal leaf tissues of seedlings where reduced levels of hexoses and TCA intermediates were observed after MeJA treatment. This is likely a consequence of the enhanced sink strength in florets compared to seedling vegetative tissues.

3.4.3. Increased accumulation of phenolics, ascorbate and leucine after MeJA treatment

With increases in shikimic acid concentrations, accumulation of phenolics was a common feature observed in all tissues after MeJA treatment. Ascorbate also responded to MeJA treatment with increased levels in broccoli tissues (Figure 3.3). Increases in these compounds elicited by MeJA treatment were also observed in *Medicago trucatula* cell cultures (Broeckling et al., 2005), aerial portions of *Arabidopsis* (Hendrawati et al., 2006), and tobacco cell cultures (Wolucka et al., 2005).

Among the various amino acids, leucine and valine exhibited significant increases in apical and basal leaves of seedlings in response to MeJA treatment (Figure 3.3A and B). Increased levels of valine, leucine and isoleucine after MeJA treatment were consistent with previous reports (Broeckling et al., 2005; van Dam and Oomen, 2008). These amino acids can be used as precursors for aliphatic GS biosynthesis (Mikkelsen and Halkier, 2003). It is not clear how amino acid biosynthetic pathways are regulated in response to MeJA treatment. Van Dam and Oomen (2008) hypothesized that Myb transcription factors may have a potential role for coordinating JA induced GS and amino acid responses.

3.5. Discussion

Changes in carbon partitioning and allocation among plant organs occurs in response to external stimuli like herbivore attack. JA treatment in *Populus tremuloides* enhanced photosynthate translocation from leaves with greater partitioning to the stem and roots (Babst et al., 2005). JA-induced young (sink) leaves imported 3-4 times as much carbon from orthostichous source leaves with a significant portion allocated into condensed tannin biosynthesis in hybrid poplar saplings (Arnold and Schultz, 2002). These results suggest that JA treatment increases the translocation of carbon resources through the induction of sink strength for the production of carbon-based defense substances (Arnold and Schultz, 2002).

Our experimental data indicate that the changes in carbon metabolism modulated by MeJA treatment are differentially regulated among seedling vegetative tissues and reproductive organs of mature plants. Increasing levels of carbohydrates in florets may be due to increased import of photosynthates from source leaves through enhanced sink strength mediated by MeJA treatment in floret tissues. Reduced levels of hexose sugars in broccoli tissues is consistent with the observations in elicitor-treated opium poppy (Zulak et al., 2007) and *Medicago truncatula* cell cultures (Broeckling et al., 2005), where increased requirements for carbon and energy is needed to support secondary metabolism (Zulak et al., 2008). Since MeJA treatment did not change photosynthetic rates in *Populus* (Babst et al., 2005), the reduced level of TCA intermediates in MeJA treated tissues may be due to enhanced carbon flux into sink tissues for downstream biochemical pathways.

Significant increases in pentose sugar levels in basal leaves of seedlings may be related to enhanced plant cell wall thickness induced by MeJA treatment as observed in tobacco (Capitani et al., 2005). Arabinose and xylose are main constituents of hemicellulose, a primary plant cell

wall component. Infection of grapevine (*Vitis*) species with downy mildew induced expression of genes involved in the pentose phosphate pathway (Polesani et al., 2010). This pathway generates NADPH and pentose sugars through two distinct phases (oxidative and non-oxidative phase) (Dennis and Blakeley, 2000). However, the relationship between pentose sugar levels and MeJA signaling is unclear. One mechanism may be that MeJA stimulates the oxidative pentose phosphate pathway to provide NADPH for NADPH:cytochrome P450 reductase reactions. Glucosinolate biosynthesis requires NADPH supply for core structure formation primarly conducted by P450s (Grubb and Abel, 2006). Further study is needed to clarify the effect of MeJA on pentose metabolism.

Consumption of broccoli with increased levels of certain phytochemicals has been associated with reduced risk of cancer and other degenerative diseases. In addition to glucosinolates, ascorbic acid and various phenolic levels were increased after MeJA treatment in our experiment. Ascorbic acid is an antioxidant that may be able to prevent oxidative damage generated from reactive oxygen and nitrogen species (Halliwell, 1996). Phenolic compounds have also been studied extensively as antioxidant protectants for humans. MeJa-mediated increases in these phytochemicals may be one of several plant mechanisms to reduce oxidative damage. More detailed information of the effect of MeJA treatments on broccoli eating and nutritional quality awaits further investigation.

Table 3.1. Groupings of metabolites based on significant differences ($P < 0.05$) in relative abundance between apical (AL) and basal leaves (BL) of seedlings, and between florets (FR) of mature plants and apical and basal leaves of seedlings. Fisher's LSD test was used to rank the tissue abundance of each metabolite between different tissue types with or without MeJA treatment.

Comparison of relative abundance	Metabolites		
	No significant changes in the rank after MeJA treatment	The rank decreased after MeJA treatment	The ranks increased after MeJA treatment
AL > BL	Fructose, Glucose, Galactose, Mannose, Glucose-6-P, Galactopyranose, Glucopyranose, Inositol, Xylitol Citric acid, Phosphoric acid, Glyceric acid, Quinic acid, Shikimic acid, Pyroglutamic acid, Chlorogenic acid, Kaempferol, Ascorbate, 3-*O*-coumaroyl-D-quinic acid Threonic acid, Glutamic acid, glutamin, Threonine, Alanine, Proline, β-alanine,	(AL > BL → AL ≤ BL) Mannose-6-P, Arabitol, Digalactosylglycerol Aconitic acid, Gluconic acid Leucine, Serine, Valine	(AL ≤ BL → AL > BL) Isomaltose, Galactitol Neochlorogenic acid GABA
BL > AL	Gulose, Sedoheptulose, Raffinose, Xylulose, Sorbitol, Erythronic acid, Ethanolamine	(BL > AL → BL ≤ AL) Ribose Galactitol	(BL ≤ AL → BL > AL) Sorbose Fumaric acid
FR > AL and BL	Sucrose, Mannose, Xylose, Glucose-6-P, Sorbose, Mannose-6-P, Galactitol, Sorbitol Gluconic acid, Ribonic acid, Glucaric acid, Hydroxylamine Glutamic acid, Glutamin, Pyroglutamic acid, Aspartic acid, Serine, Threonine, Alanine, *N*-acetylglutamic acid, Valine, Glycine, Asparagine, Methionine, Ornitine, Homoserine, Tryptophan	(FR >AL and BL → FR ≤ AL and BL) Inositol-P Fumaric acid Proline Isoleucine Tyrosine	(FR ≤ AL and BL → FR > AL and BL) Galactopyranose Pyruvic acid Ethanolamine
FR < AL and BL	Gulose, Sedoheptulose, Xylulose, Erythrose, Maltose Glycolic acid, Maleic acid, 2-Hydroxyglutaric acid, Sinapinic acid, Neochlorogenic acid, Cryptochlorogenic acid	(FR < AL and BL → FR = AL = BL) Glycerol Malic acid, Succinic acid, Lactic acid, Benzoic acid	(FR ≥ AL and BL → FR < AL and BL) Ferulic acid Shikimic acid
FR = AL = BL	Arabinose, Ribitol Stearic acid, Galactonic acid, 2-Methylbenzoic acid, Pyruvic acid, Cinnamic acid, Butylamine, Phenylalanine, Cysteine	(FR ≤ AL and BL → FR = AL = BL) Palmitic acid Caffeic acid GABA	(FR ≤ AL and BL → FR = AL = BL) Ribose, Glycerol, Digalactosylglycerol, Arabitol, Malic acid, Succinic acid, Lactic acid, Benzoic acid, Leucine (FR > AL and BL → FR = AL = BL) Inositol-P, 2-Ketogluconic acid, Isoleucine , Tyrosine (AL>FR> BL → FR = AL = BL) Aconitic acid

Table 3.2. List of the significantly increased or decreased metabolites related with carbon metabolism between different broccoli tissue types after MeJA treatment (*t*-test, $P < 0.05$).

Metabolites that increased 4 days after MeJA treatment			Metabolites that decrased 4 days after MeJA treatment		
Six weeks old seedling		Mature plant	Six weeks old seedling		Mature plant
Apical leaf	Basal leaf	Floret	Apical leaf	Basal leaf	Floret
Pentoses					
	Arabinose	Ribose			
	Xylose	Xylose			
	Xylulose				
Hexoses					
Mannose		Sorbose	Fructose	Galactose	Mannose-6-P
			Galactopyranose	Galactopyranose	
			Gulose		
			Sorbose		
			Glucose-6-P		
Disaccharides					
Maltose	Maltose	Isomaltose			
Isomaltose					
Sucrose					
Trisaccharides					
Raffinose	Raffinose				
TCA intermediates					
			Succinic acid	Succinic acid	
			Fumaric acid	Malic acid	
			Aconitic acid		
Sugar alcohols					
Inositol-P	Inositol-P	Inositol			
Galacitol	Xylitol	Xylitol			
Sorbitol	Sorbitol	Sorbitol			
Glycerol		Glycerol			
		Ribitol			

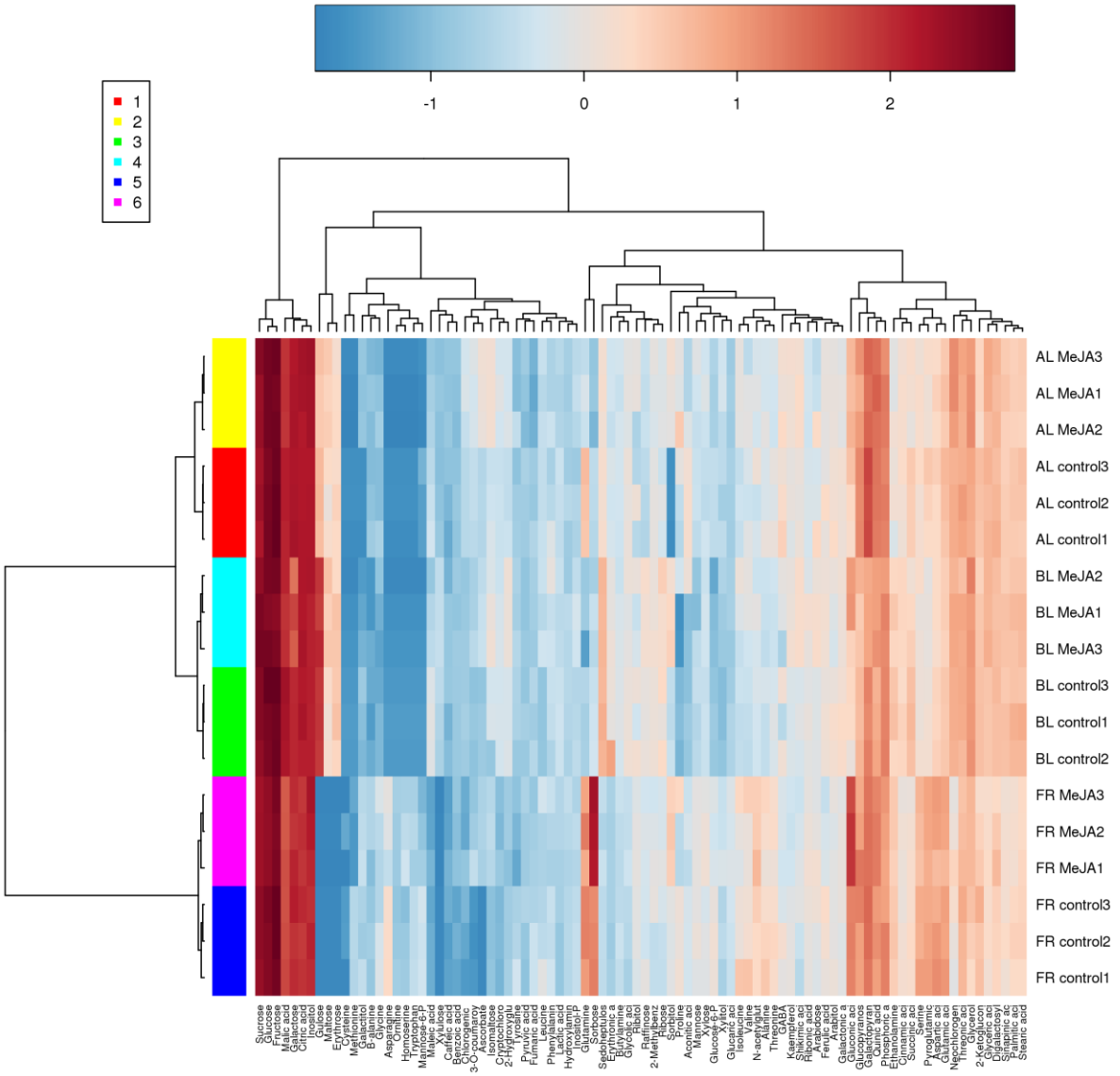


Figure 3.1. Hierarchical cluster based on tissue type (left vertical axis), apical (AL) and basal (BL) leaves of broccoli seedlings, and florets (FR) of mature plants, and changes in metabolite abundance (top horizontal axis) between the tissue types with or without MeJA treatment. The results of HCA analysis are represented as a dendrogram (distance measure using pearson and clustering algorithm using ward) and heat map to show distinct clusters observed between different tissues or among various metabolites with or without MeJA treatment. Relative abundance of metabolites is indicated by colored bars.

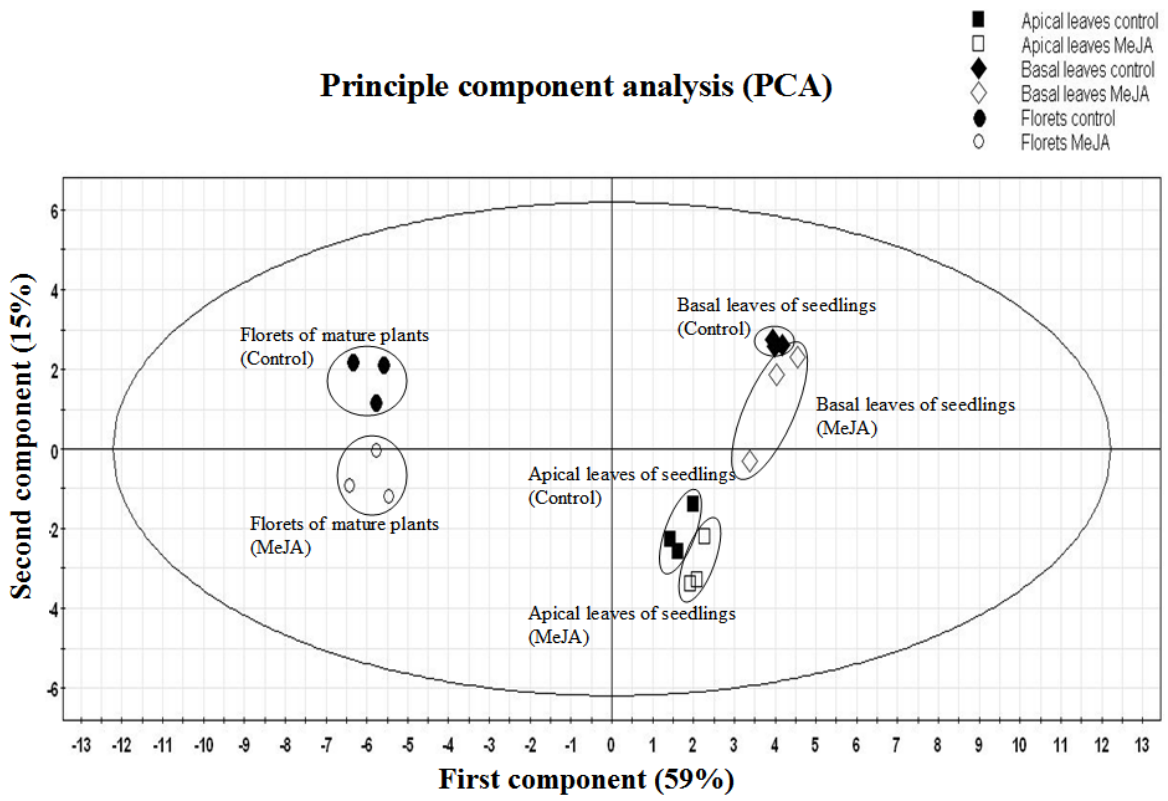


Figure 3.2. Principal component analysis (PCA) based on metabolite profiles of the apical and basal leaves of broccoli seedlings, and florets of mature plants with or without MeJA treatment.

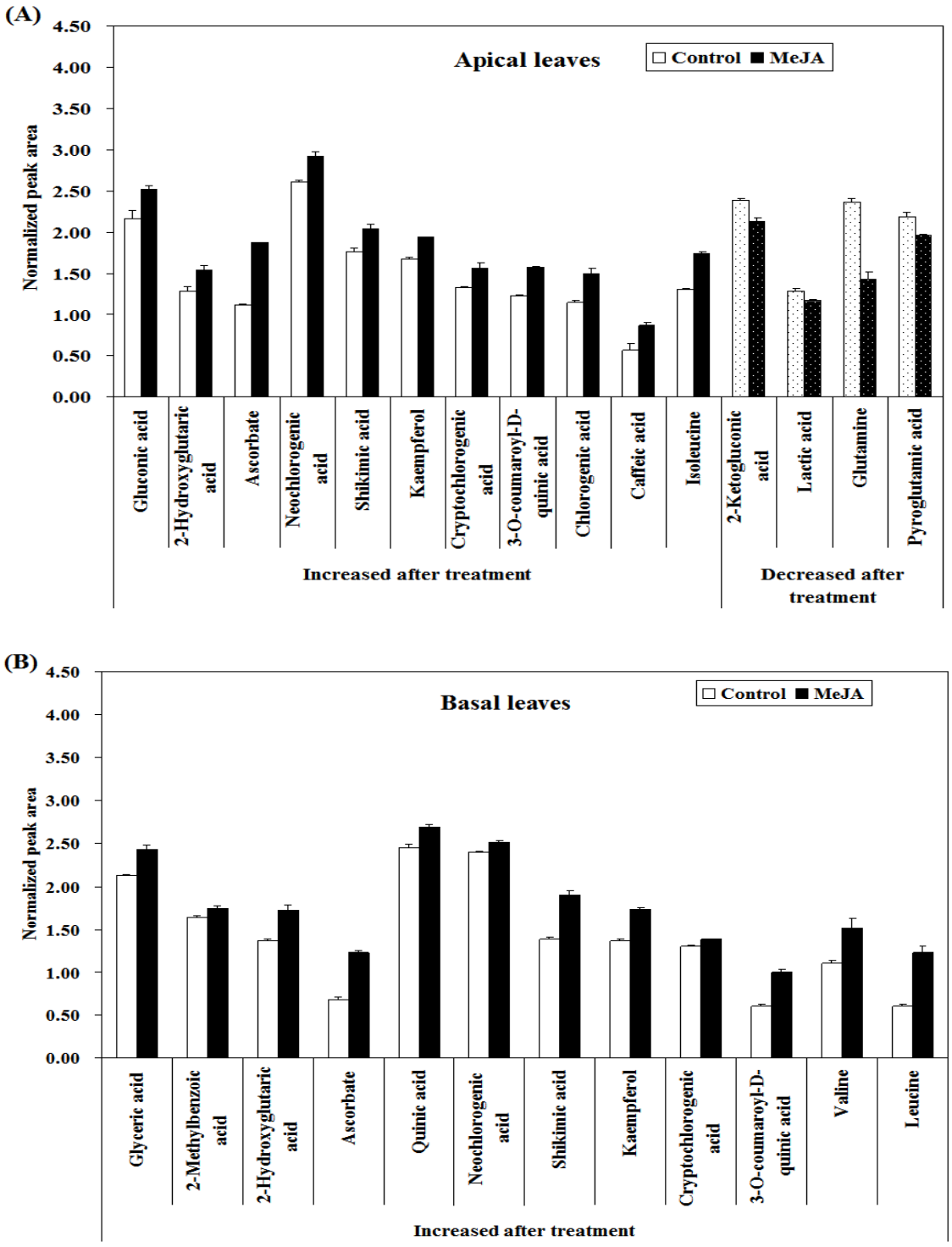


Figure 3.3. Histogram of the significantly increased (solid bars) or decreased (dotted histogram bars) organic acids, phenolics and amino acids in apical leaves (A) and basal leaves (B) of broccoli seedlings, and florets (C) of mature broccoli plants 4 days after MeJA treatment (*t* test, $P < 0.05$). Error bars represent SE of means of triplicate biological replications.

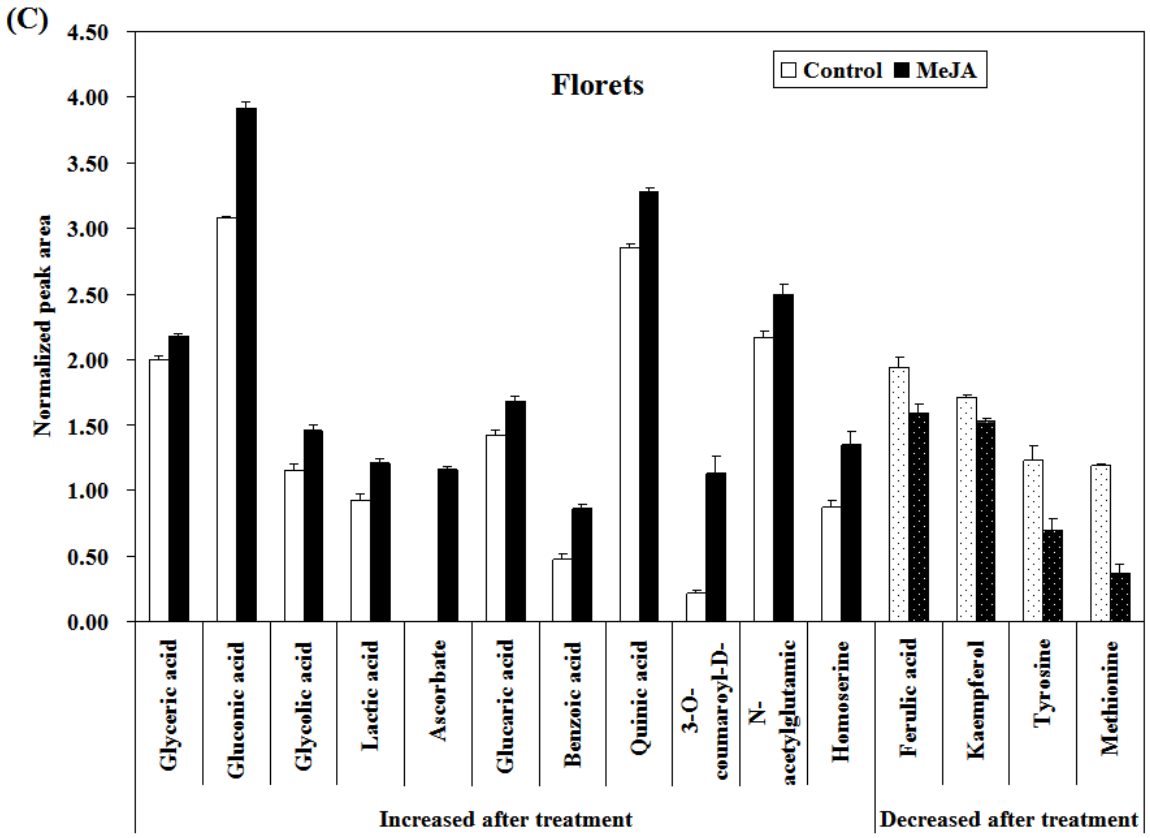


Figure 3.3. Continued.

Supplementary Table S3.1. Relative abundance of metabolite classes in apical and basal leav es of broccoli seedlings, and florets of mature broccoli plants with control or 4 days after MeJA treatment. Mean and SE of metabolite levels in arbitrary units are shown. ND indicates not determined.

No.	Metabolite	Seedlings				Mature plant	
		Apical leaves		Basal leaves		Florets	
		Control	MeJA	Control	MeJA	Control	MeJA
	Sugars						
1	Fructose	4.67±0.03	4.55±0.03	4.43±0.01	4.27±0.09	4.62±0.04	4.72±0.04
2	Glucose	4.54±0.02	4.54±0.03	4.36±0.02	4.31±0.07	4.44±0.05	4.55±0.03
3	Sucrose	4.18±0.02	4.29±0.00	4.19±0.03	4.27±0.01	4.32±0.04	4.42±0.02
4	Galactose	4.12±0.03	4.07±0.05	3.69±0.03	3.25±0.09	3.88±0.08	4.06±0.07
5	Galactopyranose	3.60±0.04	3.29±0.03	2.85±0.04	2.57±0.04	3.26±0.05	3.40±0.01
6	Glucopyranose	3.00±0.03	2.80±0.06	2.31±0.03	2.28±0.12	2.88±0.10	3.05±0.15
7	Gulose	2.47±0.01	2.36±0.00	3.54±0.02	3.61±0.06	ND	ND
8	Erythrose	2.09±0.03	2.07±0.06	2.08±0.06	2.15±0.04	ND	ND
9	Sedoheptulose	1.95±0.03	1.97±0.04	2.36±0.03	2.39±0.05	1.28±0.00	1.36±0.08
10	Maltose	1.94±0.09	2.27±0.03	1.77±0.03	2.19±0.08	ND	ND
11	Arabinose	1.71±0.19	1.58±0.24	1.42±0.01	1.83±0.01	1.72±0.08	1.90±0.09
12	Ribose	1.48±0.07	1.65±0.05	1.79±0.09	1.95±0.12	1.40±0.07	1.71±0.06
13	Isomaltose	1.40±0.06	1.96±0.03	1.10±0.19	1.65±0.04	0.80±0.02	1.30±0.01
14	Mannose	1.31±0.02	1.51±0.05	1.02±0.05	0.84±0.13	1.74±0.04	1.84±0.02
15	Xylose	1.29±0.07	1.51±0.07	1.16±0.02	1.33±0.03	1.62±0.03	1.94±0.05
16	Glucose-6-P	1.24±0.02	1.08±0.01	0.66±0.02	0.54±0.03	1.84±0.02	1.66±0.06
17	Sorbose	1.18±0.02	0.82±0.06	1.21±0.04	1.08±0.06	3.04±0.03	4.30±0.05
18	Raffinose	1.13±0.03	1.37±0.04	1.56±0.03	1.84±0.07	1.57±0.09	1.64±0.03
19	Xylulose	0.75±0.05	0.83±0.04	0.93±0.04	1.21±0.04	ND	ND
20	Maltose-6-P	0.35±0.08	0.12±0.01	ND	ND	1.45±0.04	0.90±0.10
	Alcohols						
21	Inositol	4.07±0.02	4.15±0.02	3.76±0.02	3.83±0.09	3.87±0.01	4.23±0.07
22	Glycerol	2.66±0.03	2.93±0.07	2.66±0.04	2.79±0.12	2.25±0.08	2.68±0.02
23	Digalactosylglycerol	2.52±0.05	2.55±0.03	2.24±0.01	2.30±0.05	2.19±0.03	2.44±0.11
24	Arabitol	1.94±0.06	1.80±0.04	1.72±0.05	1.55±0.12	1.76±0.04	1.81±0.05
25	Ribitol	1.36±0.19	1.56±0.18	1.54±0.14	1.46±0.15	1.66±0.03	1.81±0.03
26	Xylitol	1.12±0.09	1.42±0.06	0.53±0.03	0.88±0.10	1.14±0.04	1.55±0.07
27	Inositol-P	0.99±0.06	1.27±0.05	1.00±0.03	1.31±0.03	1.32±0.01	1.27±0.03
28	Galactitol	ND	0.87±0.03	0.57±0.07	0.34±0.04	1.12±0.09	1.37±0.05
29	Sorbitol	ND	1.84±0.03	1.10±0.04	1.98±0.02	1.67±0.03	2.40±0.03

Supplementary Table S3.1. Continued.

No.	Metabolite	Seedlings				Mature plant	
		Apical leaves		Basal leaves		Florets	
		Control	MeJA	Control	MeJA	Control	MeJA
	Organic acids						
30	Citric acid	4.06±0.03	4.08±0.03	3.84±0.04	3.90±0.05	3.80±0.06	4.00±0.01
31	Malic acid	3.99±0.02	3.78±0.04	3.92±0.01	3.68±0.08	3.56±0.03	3.67±0.04
32	Phosphoric acid	3.07±0.01	3.07±0.08	2.86±0.04	2.81±0.03	2.98±0.08	2.97±0.05
33	Glyceric acid	2.52±0.05	2.62±0.05	2.13±0.01	2.44±0.05	2.01±0.03	2.18±0.03
34	2-Ketogluconic acid	2.39±0.03	2.14±0.04	2.21±0.02	2.17±0.01	2.47±0.09	2.31±0.08
35	Stearic acid	2.32±0.04	2.25±0.02	2.31±0.09	2.31±0.03	2.16±0.04	2.26±0.03
36	Palmitic acid	2.31±0.01	2.23±0.03	2.30±0.06	2.28±0.05	2.15±0.03	2.09±0.00
37	Succinic acid	2.26±0.03	1.93±0.05	2.25±0.03	1.97±0.04	2.06±0.02	2.05±0.05
38	Gluconic acid	2.17±0.10	2.52±0.04	1.80±0.07	2.50±0.22	3.08±0.01	3.92±0.04
39	Aconitic acid	1.94±0.02	1.32±0.16	0.64±0.04	1.02±0.36	1.52±0.02	1.50±0.07
40	Galactonic acid	1.86±0.04	1.72±0.05	1.80±0.05	1.75±0.06	1.71±0.06	1.87±0.03
41	Glycolic acid	1.77±0.03	1.71±0.03	1.64±0.03	1.61±0.09	1.15±0.05	1.46±0.04
42	2-Methylbenzoic acid	1.68±0.02	1.67±0.02	1.64±0.02	1.75±0.03	1.46±0.14	1.71±0.07
43	Ribonic acid	1.51±0.00	1.48±0.03	1.51±0.03	1.66±0.06	1.78±0.03	1.96±0.05
44	Erythronic acid	1.47±0.05	1.44±0.03	1.91±0.29	1.66±0.04	1.00±0.04	1.13±0.02
45	Maleic acid	1.31±0.07	1.02±0.09	1.45±0.03	0.98±0.05	0.49±0.02	0.53±0.07
46	2-Hydroxyglutaric acid	1.29±0.05	1.54±0.05	1.36±0.03	1.73±0.06	0.99±0.02	0.98±0.05
47	Lactic acid	1.29±0.03	1.17±0.01	1.19±0.01	1.17±0.07	0.92±0.05	1.21±0.03
48	Ascorbate	1.11±0.02	1.87±0.01	0.68±0.03	1.23±0.01	ND	1.16±0.01
49	Glucaric acid	0.95±0.02	0.98±0.04	0.86±0.04	0.88±0.07	1.42±0.04	1.69±0.04
50	Pyruvic acid	0.94±0.03	0.85±0.04	0.71±0.04	0.74±0.03	0.93±0.10	1.07±0.05
51	Fumaric acid	0.93±0.08	0.57±0.04	0.89±0.06	0.88±0.06	1.31±0.07	1.04±0.05
52	Benzoic acid	0.76±0.01	0.80±0.01	0.70±0.01	0.80±0.06	0.48±0.04	0.86±0.03

Supplementary Table S3.1. Continued.

No.	Metabolite	Seedlings				Mature plant	
		Apical leaves		Basal leaves		Florets	
		Control	MeJA	Control	MeJA	Control	MeJA
	Phenolic and polyamines						
53	Quinic acid	3.17±0.03	3.28±0.06	2.45±0.04	2.69±0.03	2.86±0.03	3.28±0.02
54	Neochlorogenic acid	2.61±0.02	2.93±0.05	2.40±0.01	2.52±0.02	1.82±0.09	2.06±0.12
55	Sinapinic acid	2.23±0.02	2.31±0.04	2.17±0.04	2.22±0.02	1.98±0.04	2.07±0.02
56	Cinnamic acid	1.93±0.05	1.99±0.05	1.94±0.04	2.02±0.05	1.91±0.03	1.97±0.02
57	Ferulic acid	1.83±0.08	1.78±0.03	1.65±0.04	1.78±0.03	1.94±0.08	1.60±0.07
58	Shikimic acid	1.76±0.05	2.04±0.06	1.39±0.03	1.90±0.05	1.44±0.13	1.64±0.07
59	Ethanolamine	1.74±0.07	1.78±0.09	2.09±0.07	2.07±0.06	2.31±0.02	2.42±0.03
60	Kaempferol	1.68±0.02	1.94±0.01	1.36±0.02	1.73±0.03	1.71±0.02	1.53±0.03
61	Butylamine	1.42±0.02	1.47±0.04	1.39±0.03	1.40±0.01	1.33±0.03	1.39±0.05
62	Cryptochlorogenic acid	1.33±0.02	1.57±0.07	1.30±0.01	1.39±0.00	0.66±0.02	0.84±0.08
63	3-O-coumaroyl-D-quinic acid	1.23±0.01	1.58±0.02	0.60±0.02	1.00±0.03	0.21±0.03	1.13±0.13
64	Chlorogenic acid	1.15±0.02	1.50±0.06	0.76±0.01	0.81±0.02	0.46±0.10	0.66±0.12
65	Hydroxylamine	0.98±0.05	1.05±0.04	0.93±0.03	1.01±0.04	1.20±0.00	1.29±0.05
66	Caffeic acid	0.57±0.08	0.87±0.04	0.51±0.08	0.67±0.04	0.42±0.06	0.50±0.07

Supplementary Table S3.1. Continued.

No.	Metabolite	Seedlings				Mature plant	
		Apical leaves		Basal leaves		Florets	
		Control	MeJA	Control	MeJA	Control	MeJA
	Amino acids						
67	Threonic acid	2.72±0.03	2.68±0.01	2.39±0.04	2.46±0.04	2.59±0.06	2.60±0.04
68	Glutamic acid	2.42±0.04	2.35±0.05	2.12±0.01	2.11±0.06	2.80±0.04	2.83±0.07
69	Glutamine	2.37±0.04	1.44±0.09	0.86±0.04	0.64±0.20	2.99±0.07	3.01±0.11
70	Pyroglutamic acid	2.18±0.06	1.96±0.02	1.70±0.04	1.64±0.03	2.75±0.03	2.86±0.03
71	Aspartic acid	2.12±0.05	2.14±0.06	1.98±0.02	1.93±0.06	2.83±0.07	2.86±0.10
72	GABA	2.02±0.10	1.99±0.08	1.47±0.22	1.38±0.21	1.75±0.09	1.64±0.03
73	Serine	1.97±0.06	2.00±0.07	1.67±0.04	1.78±0.10	2.69±0.06	2.63±0.05
74	Threonine	1.69±0.07	1.51±0.01	1.37±0.03	1.30±0.06	2.07±0.03	2.11±0.05
75	Alanine	1.61±0.09	1.60±0.08	1.17±0.06	1.25±0.07	2.18±0.08	2.13±0.08
76	N-acetylglutamic acid	1.56±0.15	1.59±0.05	1.29±0.02	1.43±0.06	2.17±0.05	2.50±0.08
77	Valine	1.51±0.09	1.57±0.15	1.10±0.03	1.51±0.12	2.09±0.13	2.09±0.14
78	Proline	1.36±0.15	1.90±0.20	0.44±0.02	0.50±0.43	1.86±0.04	1.62±0.29
79	Isoleucine	1.31±0.01	1.75±0.02	1.12±0.04	1.47±0.17	2.02±0.15	1.96±0.14
80	Leucine	1.28±0.03	1.31±0.04	0.60±0.02	1.23±0.07	1.37±0.14	1.32±0.13
81	Phenylalanine	1.23±0.17	1.22±0.11	1.05±0.06	1.39±0.15	1.37±0.05	1.28±0.08
82	Tyrosine	0.86±0.06	0.94±0.18	0.81±0.05	0.99±0.05	1.23±0.11	0.69±0.09
83	Glycine	0.78±0.12	0.82±0.12	0.39±0.05	0.49±0.15	1.43±0.11	1.42±0.13
84	B-alanine	0.76±0.08	0.77±0.07	0.19±0.08	0.23±0.06	0.91±0.04	1.08±0.12
85	Asparagine	ND	ND	ND	ND	2.07±0.02	1.68±0.12
86	Cysteine	ND	ND	ND	ND	0.21±0.09	0.16±0.14
87	Methionine	ND	ND	ND	ND	1.19±0.01	0.37±0.06
88	Ornitine	ND	ND	ND	ND	0.92±0.04	1.05±0.03
89	Homoserine	ND	ND	ND	ND	0.87±0.05	1.34±0.11
90	Tryptophan	ND	ND	ND	ND	1.29±0.02	1.17±0.04

CHAPTER 4

Effect of NPA (1-naphthylphthalamic acid) on glucosinolate accumulation in *Brassica oleracea* ssp. *Italica*; Interaction of glucosinolates with the auxin transport system

4.1. Abstract

Indolyl and aromatic glucosinolates (GSs) respond to methyl jasmonate (MeJA) signaling by accumulation in broccoli tissues. The substantial increases of these compounds after exogenous treatment with MeJA in stem and petiole tissues, existence of intact indolyl-GS forms in phloem exudates, and accumulation of GSs in young apical leaves and floret tissues suggests enhanced de novo synthesis in combination with active transport. Indoly-GSs share structural similarities with the auxin, IAA, and may utilize components of the auxin transport system for intra- and extra-cellular transport. We tested the effect of an auxin transport inhibitor on MeJA-mediated accumulation of GSs in broccoli to investigate interactions between MeJA-mediated induction of tissue GS accumulation and activity of the auxin transport system. Application of auxin efflux inhibitors, 1-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), inhibited MeJA-mediated accumulation of N-methoxy-3-indolylmethyl GS in broccoli florets, whereas the auxin influx inhibitor, 1-NOA had no effect. Inhibition of NPA in up-regulation of GS biosynthesis was observed to be tissue-specific in seedlings and sprouts. NPA also inhibited the accumulation of indolyl GS following wounding. NPA did not inhibit expression of indolyl GS biosynthesis genes shown to be upregulated by MeJA treatment.

While differences were observed between aliphatic, indolyl, and aromatic GSs inhibition of IAA regulated root growth and gravitrophism in *Arabidopsis* roots, exogenous treatment of the various GSs were not as bioactive as IAA and NPA. Exogenous application of benzyl GS to *Arabidopsis* roots also induced ectopic expression of the PIN1 protein associated with the auxin

transport system similar to treatment with NPA, suggesting GS interaction with the auxin efflux carrier system. These interactions suggest that inhibitory effect of NPA on MeJA-mediated accumulation of GS may be due to competitive binding of NPA to auxin efflux carrier components and that GS translocation is mediated by the auxin transport system through direct or indirect interaction.

4.2. Introduction

In *Tropaeolum majus*, benzyl GS synthesized in leaves was observed to accumulate in developing seeds, suggesting an active translocation (Lykkesfeldt and Moller, 1993). Comparison of the GS profiles of *Arabidopsis* leaves, roots and stems from mature plants with those of immature siliques and mature seeds suggested that a majority of the seed GSs were synthesized de novo in the silique and translocated into developing seeds (Petersen et al., 2002).

Both intact GSs and desulpho-GSs possess the physico-chemical properties suggesting phloem-mediated transport (Brudenell et al., 1999). The presence of intact GSs in the phloem of *Arabidopsis* has been demonstrated, suggesting these intact GSs are the transported forms (Chen et al., 2001). To determine if GS transport follows the principle of assimilate translocation by mass flow from source to sink, Chen et al (2001) investigated GS movement in *Arabidopsis*. Radiolabelled [^{14}C] tyrosine was fed to a rosette leaf of young *35S:CYP79A1 Arabidopsis* transgenic plants, leading to the formation of radiolabelled *p*-hydroxybenzyl GS. The radiolabelled GS was exported from the leaves to the other rosette leaves, roots, stem, cauline leaves, flower buds and siliques, possibly via transport in the phloem (Chen et al., 2001).

Although the previous research suggests the existence of active transport for GSs in plants, it is still obscure as to what regulatory factors and transporter proteins are associated with systemic allocation and translocation of GSs via the phloem. Recently, a protein transporter

component of the aliphatic GS biosynthetic pathway was identified in *Arabidopsis* (Gigolashvili et al., 2009). This study found that BAT5, which belongs to the bile acid transporter gene family, acts as a plastidic transporter required for import and export of GS side chain elongation elements such as 2-keto acids and chain-elongated 2-keto acids. This result suggests that the transport of GS amino acid precursors are also important for regulation of GS accumulation in the plant.

1-naphythylphthalamic acid (NPA) is a synthetic auxin transport inhibitor (Rubery, 1990). Flavonoids including quercetin, apigenin, and kaempferol compete with NPA for binding to the NPA protein receptor in the auxin transport system and consequently perturb auxin translocation. The regulatory function of flavonoids on auxin transport was tested *in vivo* in *Arabidopsis* by comparing wild-type with a flavonoid deficient mutant (*tt4*) harboring a mutation in the gene encoding the first enzyme in flavonoid biosynthesis, chalcone synthase (Brown et al., 2001). Flavonoids have been shown to modulate auxin transport in addition to auxin-dependent tropic responses (Peer and Murphy, 2007).

The inhibiting activity of both synthetic and natural plant auxin transport inhibitors depend on the binding affinity of these compounds with components of the auxin transporter system. Structure-activity principles for auxin transport inhibitors have been proposed by Bures et al. (1991). These require an acidic functionality (e.g., carboxylic acid, acidic hydroxyl) attached to a tertiary, quaternary, or aromatic center. Among GSs, indolyl and aromatic forms have the potential to interact with auxin transporter components, since they have indolyl and aromatic side chains sharing structural similarity with IAA, NPA and PBA [2-(1-pyrenoyl) benzoic acid] (Figure 4.1). The membrane-bound efflux carriers, PIN-FORMED (PIN) proteins represent a integral part of the polar auxin transport complex (Friml and Palme, 2002). Full-

length PIN proteins, especially PIN1, are continuously cycled between the plasma membrane and endosomal compartments (Geldner et al., 2001). Their polarity can be modulated rapidly in response to developmental or external cues, thus redirecting auxin. The rate, polarity, and symmetry of the flow of the plant hormone auxin are determined by the polar cellular localization of PIN auxin efflux carriers (Santelia et al., 2008).

Indolyl GSs are rapidly induced to accumulate in tissues of *Arabidopsis, Brassica napus* and *B. oleracea* by wounding or pathogen and herbivore attack (Bartlet et al., 1999; Kim and Jander, 2007; Bednarek et al., 2009). Application of exogenous MeJA induces the production of indolyl GSs in various tissues including leaves, petioles and stems of broccoli seedings, and in the florets of mature broccoli plants (Chapter 2). Increased expression of the Trp-metabolizing gene *BoCYP79B2* after MeJA treatment was directly associated with indolyl GS biosynthesis and accumulation in broccoli (Chapter 2).

We analyzed the effect of NPA on MeJA-mediated GS accumulation in broccoli to investigate interactions between specific GS forms with components of the auxin transport system. The existence of intact GSs in phloem exudates from petioles of MeJA treated leaves of broccoli was validated using LC-ESI/MS/MS. Effect of exogenous GS compounds on IAA-regulated root growth and gravitrophism were measured in *Arabidopsis*. Potential auxin activity of GSs and direct interaction of GSs with the membrane-bound efflux carrier, PIN-FORMED1 (PIN1) protein associated with auxin transport were analyzed using *Arabidopsis* transgenic lines containing *proDR5:GUS* and *proPIN1:PIN1-GFP*, respectively. These investigations were conducted to test the hypothesis that NPA inhibits the MeJA-mediated indolyl and aromatic GS accumulation in broccoli due to competitive inhibition of interactions between components of the

auxin efflux carrier system and GSs. The degree of this competitive effect is dependent to the structural form of the GSs found in different plant organs.

4.3. Materials and methods

4.3.1. Plant material

Mature floret tissues of the broccoli cultivar 'Green Magic' were first treated with the polar auxin transport inhibitors, NPA and TIBA (auxin efflux inhibitors), and the auxin influx inhibitor 1-naphthoxyacetic acid (1-NOA), and then subjected to MeJA treatment to induce indolyl GS accumulation.

In a second experiment, six week old broccoli seedlings of two cultivars, 'Green Magic' and 'Pirate' (Asgrow Seed Co.), were subjected to NPA, MeJA, wounding, and combined treatments. These treatments were applied in a third experiment to the shoots and roots of 10 day old sprouts from germinated seed of 'Green Magic' by cotyledon rubs and supplementation of the chemical into the agar-solidified medium, respectively.

Broccoli plants at harvest maturity and seedlings were grown in the greenhouse as described in Chapter 3. Experimental design was a randomized complete block with three replicates of 3 plants spaced 30 cm apart with 30 cm between rows on greenhouse benches. In the third experiment, broccoli seeds were sterilized by immersion in 0.5% NaOCl solution for 15 min, germinated on MS basal medium (Murashige & Skoog 1962), and grown in a tissue culture room under the 16 hr of cool white fluorescent light (70 μE μmol $m^{-2}s^{-1}$ of photosynthetically active radiation) at 24 ± 2 °C. Experimental design was a randomized complete block with three replicates of 25 sprouts.

4.3.2. MeJA, wounding and polar auxin transport inhibitor treatment

In the first experiment, all aerial portions of mature broccoli plants were sprayed with 250 µM MeJA (Sigma-Aldrich, St. Louis, MO), 200 µM NPA (Pfaltz & Bauer, West Chester, PA), 50 µM TIBA (Sigma-Aldrich), 50 µM 1-NOA (Sigma-Aldrich), and the combined treatments of 250 µM MeJA with each polar auxin transport inhibitor. In the second experiment, aerial portions of six-week old broccoli seedlings were sprayed with 250 µM MeJA, 200 µM NPA, and a combination of both MeJA and NPA. Control plants were sprayed with water containing 0.1% Triton X-100 and equal amount of solvent, 0.02% dimethyl sulfoxide (DMSO) (Sigma-aldrich) used with the auxin inhibitor treatments. Plants were spray treated in isolation and allowed to dry prior to being returned to greenhouse benches. For the combination treatment of polar auxin transport inhibitors with MeJA or wounding, plants were treated with the polar auxin transport inhibitors first, allowed to dry, and then treated with MeJA or subjected to wounding. For the wounding treatment, the fourth and fifth basal leaves from the apex of six week old seedlings were subjected with the wounding device as described in Chapter 2. Floret tissues of mature broccoli and the different tissues in broccoli seedlings and were harvested 1, 2 and 4 days after treatments. In six-week old seedlings, apical (second and third leaves including emerging shoots from the apical meristem) and basal (4th-6th node) leaves, petioles of basal leaves, and stem tissues from each plant were harvested. Three plant subsamples were bulked to comprise each of the three replicates, frozen in liquid nitrogen, ground into powder, and stored at -80 ℃ for RNA extraction and a portion lyophilized and stored at -20℃ for HPLC analysis.

In the third experiment, 3 day old germinating broccoli seedlings were transferred onto medium supplemented with 20 µM NPA, 50 µM MeJA, or a mixture of both chemicals. Seedlings were dissected and harvested into root and shoot subsamples by separation at mid-

hypocotyl 7 days after treatment. For the shoot treatments, the cotyledon of 6-day old broccoli sprouts were sprayed with 200 µM NPA, 250 µM MeJA, or a mixture of both chemicals followed by rubbing the cotyledon with sterilized cotton Q-tips. Shoot and root subsamples were harvested 4 days after treatments. Control sprouts were treated by supplementation of an equal amount of DMSO into the medium and cotyledon rubbing. Equal subsamples of tissues from each plant were bulked to comprise each of the three replicates, frozen in liquid nitrogen, lyophilized, ground into powder and stored at -20℃ until HPLC analysis.

4.3.3. Glucosinolate analysis

GSs in lyophilized tissues were extracted and analyzed by high-performance liquid chromatography (HPLC) using a reverse phase C^{18} column as described by Kim and Juvik (submitted). GSs were desulfated with sulfatase in solution (Sigma-Aldrich) in columns containing DEAE Sephadex A-25 resin (Sigma-Aldrich), and eluted desulfo-GSs were separated on a HPLC system consisting of a DIONEX GP40 gradient pump with a AD20 UV absorbance detector (Sunnyvale, CA) set at 229 nm wavelength, an auto-sampler, an all-guard ™ cartridge guard column (Alltech, Lexington, Kentucky), and a Lichosphere 100 RP-18 column (Grace Co. Deerfield, IL). The type and amount of GSs in each sample were calculated in comparison to certified glucosinolate levels in a standard rapeseed reference material (BCR 367, Commission of the European Community Bureau of References, Brussels, Belgium). GSs were quantified with benzylglucosinolate (POS Pilot Plant Corp, Saskatoon, SK, Canada) as an internal standard using UV response factors for different forms of GSs determined by Wathelet et al (2001). The identification of intact and desulfo-GS profiles were validated by LC-tandom MS using a Waters QT of Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC-MS using a Finnigan LCQ Deca XP, respectively. The molecular ion and fragmentation patterns of

individual intact and desulfo-GS were matched with the literature for GS identification and validation (Tian et al., 2005; Barbieri et al., 2008).

4.3.4. Statistical analysis

GS concentrations obtained from broccoli tissues treated with different chemicals were analyzed by the GLM procedure and Fisher's LSD tests using the SAS statistical analysis package (SAS version 8.0).

4.3.5. Free tryptophan analysis

Free tryptophan levels in apical leaf tissues of broccoli seedling subjected to control, MeJA, NPA and combination treatment of MeJA and NPA were analyzed by HPLC using a Adsorbosil C^{18} column (Alltech Associates, Deerfield, IL) and fluorescence detection (Kratos FS970; excitation, nm; emission, band pass filter > 375 nm) as described by Cho et al. (2000). Free tryptophan in lyophilized tissues was extracted with 0.1 N HCl, and the extract was deproteinated using an UltraFree-MC (10,000) filter unit (Millipore, Bedford, MA).

4.3.6. Quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was performed using SYBR® Green PCR Master Mix (Applied Biosystems) in the ABI PRISM/Taqman 7700 Sequence Detection System (Applied Biosystems). Total RNA was extracted using the RNeasy Mini Kit with RNase-Free DNase (QIAGEN). cDNA was synthesized from 1 μg of the total RNA using Superscript® III First-Strand Synthesis System with the mixture of random hexamer and oligo-(dT) primer (Invitrogen, Carlsbad, CA). Target genes and primer sequences used for our experiment were described in Chapter 6. Broccoli *Actin-1* was used as the normalizer (reference) gene. All reactions were run in triplicate from the corresponding three biological replications, and ratio calculations were conducted by the mathematical model proposed by Pfaffl (2001) using ΔCt

(the cycle threshold value difference between control and sample of target and reference gene transcript).

4.3.7. Detection of intact GSs in phloem exudate using LC-ESI/MS/MS

Phloem exudate was collected from basal leaves (4th-6th node) of broccoli seedlings using the EDTA method (King and Zeevaart, 1974; Chen et al., 2001). Basal leaves were excised, and the basal part of the petioles recut under water with subsequent washing. The basal leaves were incubated in vials containing 10 ml of 20 mM EDTA (pH 7.7) for 4 h in a humid chamber at 20 ℃. Phloem exudates were obtained from the plants one day after treatment of control, MeJA, NPA and the combined treatment of NPA and MeJA as described in the second experiment. Phloem exudates collected from the basal leaves of three plants were combined in each of three replicates, lyophilized and stored at -80 ℃. Fresh weight of basal leaves in each replication was measured for normalization of peak area obtained from LC-ESI/MS/MS.

Analysis of intact GSs in phloem exudates was performed using a Waters QT Ultima spectrometer coupled to a Waters 1525 HPLC system in the Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign. HPLC separation and negative ion tandem mass spectrometry (MS/MS) using multiple reaction monitoring were conducted as described by Tian et al. (2005). 2-Propenyl GS (sinigrin) (Sigma-Aldrich) producing maximum abundant precursor ion *m/z* 358 ($[M-H]^-$) and fragment ion *m/z* 97 ($[SO3H]^-$) signals during MS/MS was used to tune the mass spectrometer. The following transitions were used to detect 10 individual GSs: 3-methylsulphinylpropyl GS (422 > 97), 2-hydroxy-3-butenyl GS (388 > 97), 4-methylsulphinylbutyl GS (436 > 97), 2-propenyl GS (358 > 97), 3-butenyl GS (372 > 97), 3-indolymethyl GS (447 > 97), 5-methylsulphinylpentyl GS (450 > 97), 4-methoxy-3-

indolymethyl GS (477 > 97), 2-phenylethyl GS (422 > 97), and N-methoxy-3-indolylmethyl GS (477 >97) (Tian et al., 2005; Skutlarek et al., 2004).

4.3.8. *Arabidopsis* root growth and gravitrophism in response to GS exposure

The effect of different GSs on *Arabidopsis* root growth and gravitropism were measured in agar medium. N-methoxy-3-indolylmethyl GS, benzyl GS, and 2-propenyl GS were selected as the representative of indolyl, aromatic and aliphatic GSs, respectively (Figure 4.1).

N-methoxy-3-indolylmethyl GS was purifed from MeJA treated broccoli floret tissues as described by Truscott et al. (1983) and Clay et al. (2009) with modification. Methanol extract obtained from 20 g of freeze-dried tissues were filtered, deprotenized, and loaded into an 8 x 1 cm column of charged DEAE Sephadex A-25 resin. Aliphatic GSs were washed with 40 ml of 0.125 M pyridine acetate, and subsequently washed with 10 ml of distilled H_2O. Indolyl GSs were eluted from the column by washing with 0.3 M potassium sulphate. Separated fractions of each sample were collected, desulfated, and analyzed by HPLC as described above. Purity and validation of N-methoxy-3-indolylmethyl GS in samples that were not desulphated was determined by LC-MS at >90%. Each fraction containing N-methoxy-3-indolylmethyl GS was combined, mixed with 100% ethanol to precipitate the potassium sulphate salt, centrifuged, evaporated, and lyophilized. During the lyophilization, the moisture and water generated inside of chamber were frequently removed.

IAA, IAN, NPA and MeJA were also tested for effects on *Arabidopsis* root growth to compare with those of GSs. Different concentrations ranging from 10 nM to 1 mM were added into the agar medium, and all medium including control plates standardized to contain 0.2% DMSO and ethanol as a final concentrations. *Arabidopsis thaliana* seeds of the Columbia ecotype were sterilized, placed on half strength MS medium containing 1% sucrose and 1% agar,

stored for 2 days at 4 ℃ in the dark, and then placed in a tissue culture room under continuous cool white fluorescent light (70 μE μmol $m^{-2}s^{-1}$ of photosynthetically active radiation) at 24 ± 2 °C. Four-day old seedlings were transferred on the agar plates containing different concentrations of chemicals. Root tips were aligned for the measurement of root tip curvature and root length. After 24 h, plates were turned 90°, and the angle of gravitropic curvature was measured after another 24 h. For the measurement of root length, plates were held in a vertical position for 48 h after root alignment. Six seedlings were tested in each plate with three replications.

4.3.9. GUS expression analysis

Potential auxin activity generated from application of exogenous GS compounds was tested in the *Arabidopsis thaliana* double mutant, *sur2 DR5:GUS*, obtained from the Arabidopsis Biological Resource Center (ABRC) (Stepanova et al., 2005). The mutation in *CYP83B1* (*sur2*) is an indolyl GS knock out mutation and was used to remove the potential effect of endogeneous GSs induced by the up-regulation of upstream genes in the indolyl GS biosynthetic pathway. Four-day old seedlings were transferred into half strength MS medium containing 1% sucrose and 1% agar supplemented with 1 μM IAA, 20 μM NPA, 20 μM MeJA, 20 μM benzyl GS, 40 μM N-methoxy-3-indolylmethyl GS, or 500 μM 2-propenyl GS, and incubated under continuous light in the tissue culture room for 48 h.

Histochemical GUS analysis was performed using the procedure described by Jefferson et al. (1987). *Arabidopsis* seedlings were immersed in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 100 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.1% Trition X-100, and incubated at 37°C for 6 h. Tissue chlorophyll was removed by immersion in 70% ethanol. Individual representative

samples were photographed using an MRc 5 color camera connected with Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY).

4.3.10. GFP localization analysis

The effect of exogenous treatment of GSs on PIN1 localization was analyzed in the *Arabidopsis* transgenic line, *PIN1:GFP* producing a PIN1:GFP fusion protein. *PIN1:GFP* seeds were obtained from the ABRC (Benková et al., 2003). Four-day old seedlings were placed on agar medium supplemented with 1 μM IAA, 20 μM NPA, 20 μM MeJA, 100 μM benzyl GS, 500 μM N-methoxy-3-indolylmethyl GS, and 500 μM 2-propenyl GS, and incubated under the same conditions as described above. GFP was visualized using Zeiss 710 laser confocal scanner (Carl Zeiss). Identical settings for laser, pinhole and gain of the confocal microscope were used for all sample analyses.

4.4. Results

4.4.1. Auxin efflux inhibitors, NPA and TIBA, inhibited MeJA-mediated indolyl GS accumulation in broccoli

The auxin efflux inhibitors, NPA and TIBA, and the auxin influx inhibitor, 1-NOA, were applied in combination with MeJA to analyze the effect of these treatments on MeJA-mediated GS accumulation in broccoli florets of 'Green Magic' (Figure 4.2). Previous experiments indicated that the application of exogenous MeJA increased indolyl GS concentrations up as much as 5-fold in broccoli floret tissues (Chapter 2). Combined treatment of NPA or TIBA with MeJA significantly decreased concentrations of N-methoxy-3-indolylmethyl GS compared to MeJA application alone 1 day after treatment. The inhibitory effect of NPA was transient, where by 2 days concentrations of the N-methoxy-3-indolylmethyl GS were not significantly different between the two treatments (Figure 4.2). NPA or the combined treatment of NPA with MeJA did

not show any significant effects on other aliphatic or aromatic GS concentrations in floret tissues (data not shown). The auxin influx inhibitor, 1-NOA, and MeJA combined treatment did not show significant differences in accumulated levels of N-methoxy-3-indolylmethyl GS in floret tissues compared with MeJA treatment alone (Figure 4.2). Only the auxin efflux inhibitor interfered with MeJA-mediated induction of N-methoxy-3-indolylmethyl GS biosynthesis.

Differential responses among broccoli genotypes to the MeJA treatment may generate the variation in induced levels of N-methoxy-3-indolylmethyl GS after treatment. To test this and to validate the inhibiting effect of NPA on indolyl GS accumulation, NPA was applied to six-week old broccoli seedlings with combined treatments of MeJA or basal leaf wounding in two broccoli cultivars 'Green Magic' and 'Pirate' (Figure 4.3). The level of N-methoxy-3-indolylmethyl GS in apical and basal leaf tissues of the two genotypes subjected to the combined treatment of MeJA and NPA were significantly lower than those in plants treated solely with MeJA 2 days after treatment (Figure 4.3 A). Petiole and stem tissues of broccoli seedlings also exhibited a similar pattern of GS reduction in concentration when compared with those from MeJA treatments alone (Figure 4.3 B). The inhibitory effects of NPA were observed in all plant tissues analyzed. NPA also inhibited the accumulation of indolyl GS induced by wounding (Figure 4.3 C).

4.4.2. Tissue-specific inhibitory effects of NPA on indolyl and aromatic GS accumulation

GS accumulation with NPA or combined treatments of NPA with MeJA was analyzed in 10-day old broccoli sprouts (Figure 4.4). The dominant GS form in root tissue of broccoli sprouts was the aromatic GS, 2-phenylethyl GS. Exposure of MeJA to root tissue through the supplementation of MeJA into the agar medium resulted in an increase of indolyl and aromatic GS concentrations in both the shoot and root portions of sprout hypocotyls. The combined

treatment demonstrated the inhibitory effect of NPA on MeJA-mediated accumulation of both 2-phenylethyl GS and N-methoxy-3-indolylmethyl GS in the root portion of the sprouts (Figure 4.4). The concentration of 2-phenylethyl GS was significantly lower in root tissues subjected to only NPA compared to controls, suggesting that NPA inhibits not only MeJA-mediated 2-phenylethyl GS accumulation but also accumulation of endogenous 2-phenylethyl GS presumably generated through *de novo* biosynthesis in root tissues. Combined application of NPA and MeJA into the root tissues did not inhibit the accumulation of indolyl GSs in the shot portions of sprouts compared to controls. Shoot application of NPA through cotyledon rubbing interfered with MeJA-mediated accumulation of N-methoxy-3-indolylmethyl GS only in the shoots with no observed inhibition in root tissues (Figure 4.4). NPA apparently inhibits the accumulation of indolyl and aromatic GSs only in tissues directly treated with NPA.

4.4.3. Effect of NPA on free tryptophan levels in apical leaves of broccoli seedlings

What other metabolic processes might be responsible for the observed effect of NPA on MeJA-mediated accumulation of indolyl GS? One possibility is that NPA is affecting the availability of indolyl GS precursors. Tryptophan is an amino acid precursor of indolyl GS (Bak and Feyereisen, 2001). Application of MeJA has been observed to increase the levels of tryptophan in *Arabidopsis thaliana* (Liu et al., 2010). To test if NPA interferes with the accumulation of tryptophan, consequently inhibiting *de novo* biosynthesis of indolyl GSs due to limiting availability of the amino acids precursor, free tryptophan levels were analyzed in apical leaves of broccoli seedlings subjected to control, MeJA, NPA or a combined treatment of NPA with MeJA (Figure 4.5). The combined treatment of NPA with MeJA did not show significant differences in free typtophan levels compared with MeJA treatment alone. This result suggests

that the inhibitory effect of NPA on MeJA-mediated accumulation of indolyl GS is not due to limiting the pool of indolyl GS amino acid precursors.

4.4.4. NPA does not inhibit the expression of genes associated with GS metabolism

The inhibitory effect of NPA may also be due to a direct or indirect suppression of indolyl GS biosynthesis gene expression. To test this, expression levels of genes involved in GS metabolism were analyzed in seedling apical leaves and floret tissues of mature plants subjected to control, NPA, MeJA and the combined treatment of NPA with MeJA by qRT-PCR (Figure 4.6). Gene expression of the indolyl GS biosynthesis genes, *BoCYP79B2* and *BoCYP83B1*, and genes involved in GS hydrolysis, *BoMYO* and *BoESP*, was comparable in the transcript abundance observed in apical leaves of seedlings and floret tissues subjected to MeJA treatment, suggesting that NPA does not directly influence the gene regulatory pathway in indolyl GS biosynthesis. The NPA effect on indolyl glucosinolate accumulation is not observed at the gene expression level.

4.4.5. N-methoxy-3-indolylmethyl GS is the dominant GS form in phloem exudates

N-methoxy-3-indolylmethyl GS levels increased by 4.2 and 7.1 fold after 2 days of MeJA treatment in the petiole and the stem tissues of Green Magic genotype whereas combined MeJA and NPA treatments increased only 1.7 and 3.3 fold, respectively (Figure 4.3 B). This result suggests that NPA affects translocation of N-methoxy-3-indolylmethyl GS through the phloem. Increased levels of indolyl GS content in the petiole and stem tissues after MeJA treatment may be due to the loading of these compounds from source leaves into the phloem. The presence of intact GSs in the phloem has been observed in *Arabidopsis* by the analysis of desulfated-GSs using LC-MS (Chen et al., 2001).

Phloem exudates were extracted from the petiole of basal leaves 1 day after application of NPA, MeJA or the combined treatment of NPA with MeJA and analyzed using LC-ESI/MS/MS. The dominant GS in phloem exudates was N-methoxy-3-indolylmethyl GS, suggesting this compound is found in the phloem in the intact GS form (Figure 4.7 A). This result is consistent with the observation of Chen et al. (2001) in *Arabidopsis*. However, the relative abundance of this compound did not show significant differences among the samples in different treatments (Figure 4.7 B). Wounding that occurred during the petiole excision also may stimulate increase in N-methoxy-3-indolylmethyl GS concentration in phloem exduate. Otherwise, the inhibiting effect of our NPA treatment on translocation of N-methoxy-3-indolylmethyl GS through the phloem may not be strong enough to show discriminative levels of this compound among different treatments in this tissue.

4.4.6. Effect of exogeneous GSs on *Arabidopsis* root gravitropism

Our GS profiling data suggest that MeJA-mediated accumulation of N-methoxy-3-indolylmethyl or 2-phenylethyl GS in broccoli tissue may be related with components of the auxin efflux carrier system by competitive carrier protein binding. If the specific forms of GS share structural chemical homology to auxin they could directly or indirectly interact with polar auxin transporter components, which would be reflected by the changes in root gravitrophism, a cellular process under auxin regulation. The differential growth response associated with gravitrophism occurs in the elongation zone of the root through asymmetric distribution of auxin to the lower side of epidermal cells (Moore, 2002; Muday, 2001; Chen et al., 1998). In the tissue accumulating auxin, cell elongation is inhibited and the root tip bends downwards. This cell-to-cell or polar auxin transport is determined by asymmetric cellular localization of auxin in- and

efflux components (Vieten et al., 2007; Wisniewska et al., 2006; Geisler and Murphy, 2006; Kerr and Bennett, 2007).

Different concentrations of 2-prophenyl (aliphatic GS), N-methoxy-3-indolylmethyl (indolyl GS) or benzyl GS (aromatic GS) ranging from 10 nM to 1 mM were tested to analyze the effect of these compounds on root growth and gravitrophism in *Arabidopsis* and compared with the effects with IAA, IAN, NPA and MeJA (Figure 4.8). For the aromatic GSs, benzyl GS, structurally similar to 2-phenylethyl GS was used instead of 2-phenylethyl GS due to difficulties in purification of this aromatic GS. Application of 100 μM benzyl and N-methoxy-3-indolylmethyl GS reduced *Arabidopsis* root growth by approximately 95%, whereas the aliphatic GS had no effect on root growth at same concentration (Figure 4.8 A). Overall inhibitory activities of GSs on *Arabidopsis* root gravitrophism were not as pronounced as IAA and NPA. However, benzyl GS exhibited a relatively strong reduction in gravitrophic response compared to N-methoxy-3-indolylmethyl and 2-prophenyl GS which showed intermediate and lowest levels of inhibition at 100 μM of treatment, respectively (Figure 4.8 B). These results suggest that exogenous aromatic and indolyl GS inhibit auxin transport activity, but require higher concentrations compared to NPA.

4.4.7. Auxin activity generated through the potential hydrolysis of endogenous GSs

The auxin activity potentially generated from endogeneous GS hydrolysis could interfere with the putative inhibitory interaction between exogeneous GS and auxin transport. The hydrolysis of indolyl GSs form indolyl-3-acetonitrile. This nitrile has auxin activity, and could be converted to indole-3-acetic acid by nitrilase enzymes (reviewed in Agerbirk et al., 2009). Potential auxin activity generated from the exogeneous GS treatment was measured using *DR5-GUS* which responds to the changes in auxin-induced gene expression (Ulmasov et al., 1997). To

exclude the potential effect of endogeneous GSs, *DR5-GUS* was introgressed into the *cyp83b1* (*sur2*) mutant background (Stepanova et al., 2005). Application of MeJA or IAA produced intense staining in the whole area of *Arabidopsis* root, as expected, while exogenous NPA, benzyl GS, N-methoxy-3-indolylmethyl, and 2-prophenyl GS stained only the root tip (Figure 4.9). This result indicates that auxin activity hypothetically generated by GS hydrolysis is a negligible in our experimental procedures.

4.4.8. Application of exogenous benzyl GS mediates ectopic expression of PIN1 in *Arabidopsis thaliana* roots

If N-methoxy-3-indolylmethyl and 2-prophenyl GS compete for the same binding site with NPA in the auxin efflux carrier complex, and act as a regulatory subunit for auxin efflux, exogenous treatment of N-methoxy-3-indolylmethyl and 2-prophenyl GS also may show similar patterns of PIN1 localization in *Arabidopsis* primary roots as previously observed with NPA treatments (Benková et al., 2003; Peer et al., 2004; Sauer et al., 2006). Based on the concentrations need for 50% inhibition of *Arabidopsis* root growth (IC_{50}) (Figure 4.8 A), *proPIN1:PIN1-GFP Arabidopsis* transgenic roots were treated with 1, 20, and 20 μM of IAA, NPA and MeJA, respectively. For treatment of benzyl, N-methoxy-3-indolylmethyl and 2-prophenyl GS, the approximate IC_{50} concentrations for root gravitrophic inhibitory response were employed.

In the controls, PIN1 was localized at the stele, pericycle and endodermis cells (Figure 4.10). Treatment of exogenous IAA and NPA relocated PIN1 into cortex and epidermis cells with wide-spreading ectopic expression of PIN1 in the epidermis cells. Exogenous benzyl GS exhibited a pattern of ectopic expression similar to NPA treatment (Figure 4.10). These results indicate that benzyl GS interacts with auxin efflux carrier components, and these interactions

may be associated with the regulatory mechanism of GS and auxin transport in the plant. MeJA, N-methoxy-3-indolylmethyl and 2-prophenyl GS did not show as dramatic differences in PIN1 relocalization compared with the control treatment.

4.5. Discussion

4.5.1. NPA-mediated inhibition of GS accumulation in broccoli

Increases in N-methoxy-3-indolylmethyl GS concentrations (from 0.20 to 17.70 μM/g of dry weight in control versus treated plants) in response to MeJA treatment in broccoli provides a system to investigate the relative roles of *de novo* biosynthesis and translocation in GS accumulation and distribution. Previous research conducted in *Arabidopsis* using the same MeJA treatment protocol resulted in increases of N-methoxy-3-indolylmethyl GS from 0.3 to 3.8 μM/g of dry weight (Mikkelsen et al., 2003).

NPA and TIBA have been used extensively as synthetic auxin transport inhibitors (Rubery, 1990). Although both compounds inhibit polar auxin transport by inhibiting auxin efflux, they have different membrane binding features (Hertel et al., 1972; Katekar and Geissler, 1977; Brunn et al., 1992). NPA does not compete with IAA for a common binding site, whereas TIBA competes for binding. Our experimental data demonstrated that both NPA and TIBA have inhibitory effects on MeJA-mediated accumulation of N-methoxy-3-indolylmethyl GS in broccoli florets. This result suggests that competition between NPA and GSs for binding to the NPA protein receptor or other auxin efflux components with IAA binding sites in the auxin transport system may be associated with the decreased accumulation of N-methoxy-3-indolylmethyl GS induced by stimulation of MeJA in broccoli floret tissues. Combined treatment of NPA with MeJA did not influence MeJA-mediated expression of indolyl GS biosynthesis

genes or free tryptophan levels, indicating that tissues subjected to the combined treatment still maintained indolyl GS biosynthetic capability comparable to controls.

It is not clear what kind of regulatory mechanisms are involved with the NPA-mediated inhibitory effect on induction of indolyl and aromatic GS in MeJA treated tissues. Indolyl GS is an important class of tryptophan (Trp) secondary metabolites which share precursors with the auxin biosynthetic pathway (reviewed in Zhao, 2010). Indolyl GS can act as precursor to IAA through hydrolysis and conversion by the action of nitrilases. These similarities in metabolism between indolyl GS and IAA address the possibility that they may share the same efflux components required for transport and vacuolar trafficking. Auxin efflux carrier components, PIN proteins, constitutively cycle between the plasma membrane and endosomes (Ljung et al., 2002). Recent research has demonstrated that the vacuolar H^+-pyrophosphatase, AVP1, traditionally considered as a vacuolar H^+-pump, also functions in modulating auxin transport (Li et al., 2005). GSs are synthesized in the cytosol with P450 enzymes involved in core structure formation, and stored in vacuoles of most plant tissues (Kelly et al., 1998; reviewed in Grubb and Abel, 2006). If NPA competitively binds to an unknown GS transporter complex in vacuolar membranes (tonoplasts) with indolyl or aromatic GSs, it may inhibit the sequesteration of newly synthesized cytosolic indolyl or aromatic GSs into the vacuole. This hypothetical inhibitory process may stimulate the turnover of GSs, potentially leading to breakdown of GS compounds in the cytosol through recycling and hydrolysis.

NPA also may inhibit the extracellular transport of these compounds through the efflux carrier proteins in the plasma membrane. P-glycoprotein (PGP)-type ATP-binding cassette (ABC) transporters, which hydrolyze ATP to transport substrates, have been shown to interact with flavanols, natural polar auxin transport inhibitors (Geisler et al., 2005; Lewis et al., 2007;

Wu et al., 2007). ABC transporter families are involved with the membrane transport of endogenous secondary metabolites in plants (Yazaki, 2006). This result suggests that components of the auxin transport system may be related with extra or intercellular transport of metabolites which have binding affinity with the auxin efflux carrier proteins.

4.5.2. Potential role of the interaction between GS and auxin efflux carrier in the plant

Jasmonates play major roles in plant defense through the activation of genes related with the synthesis of defense substances in response to abiotic and biotic stresses (Pauwels et al., 2008). Indolyl GSs are compounds rapidly induced by jasmonate signaling in *Brassica* species (van Dam et al., 2003). Our previous experiments showed that indolyl GS accumulation by MeJA treatment was primarily observed in the sink organs such as young apical leaves and florets of broccoli (Chapter 2). Substantial increases of these compounds in stem and petiole tissues with MeJA treatment suggest a response by active de novo synthesis in source tissues with transport of these compounds to sink tissues. GS profiles obtained from roots of broccoli sprouts and seedlings responded to MeJA primarily by synthesis of 2-phenylethyl GS. This organ-specific distribution and accumulation of distinct classes of GSs suggest that these compounds play different roles in plant defense.

The inhibitory effect of NPA on indolyl and aromatic GS accumulation and the bioactivity of exogenous treatment of these GS compounds in PIN1 localization, *Arabidopsis* root growth, and gravitrophic response suggest that indolyl and aromatic GSs may be antagonistic to IAA transport, biosynthesis and activity. Indolyl and aromatic GSs can also be potentially converted into IAA by hydrolysis. This intrinsic feature of GSs may be the part of a sophisticated regulatory process where the metabolic pathways in the plant shift from active growth to a reversible defense posture in response to biotic or abiotic stress. It seems likely that

indolyl and aromatic GSs are important compounds that provide connections between jasmonate and auxin singaling. Further studies are required to reveal the regulatory mechanism for crosstalk between the two hormones.

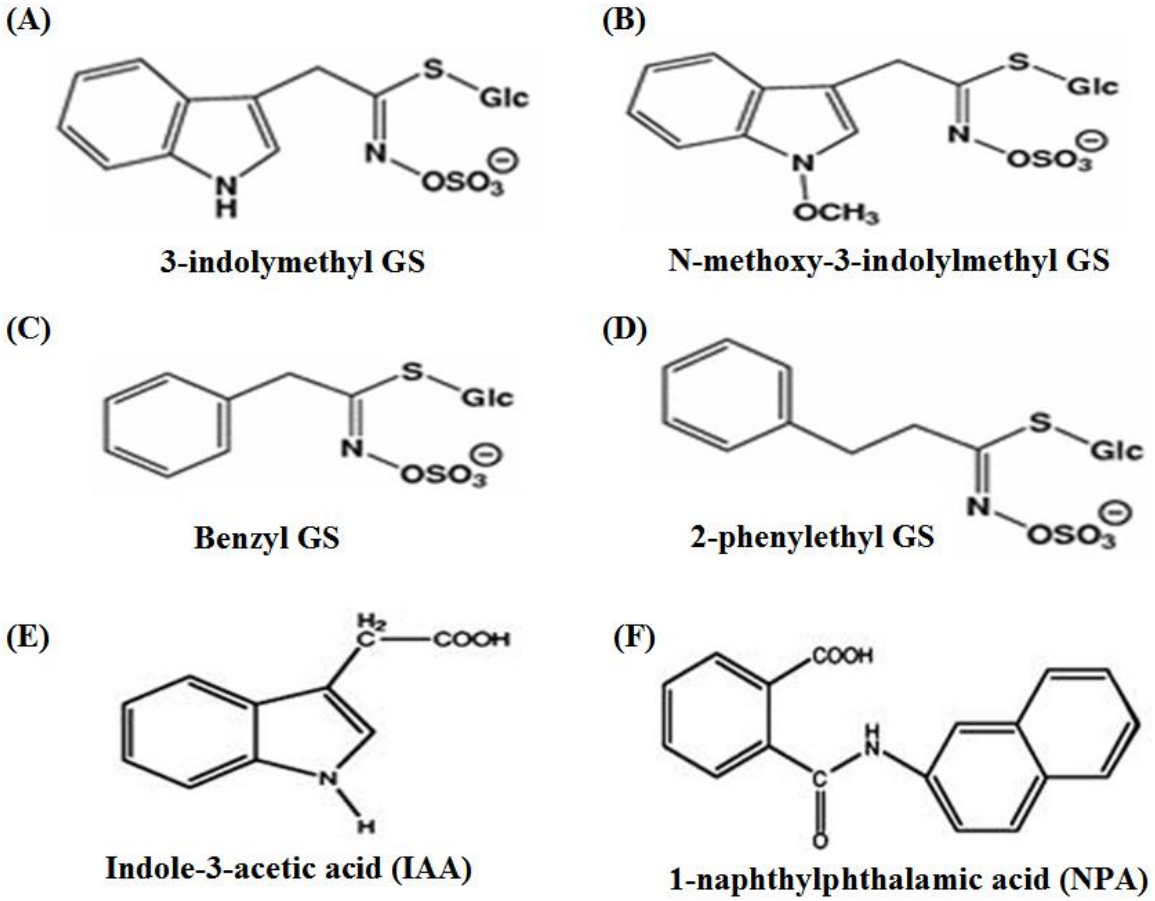


Figure 4.1. Chemical structures of indolyl and aromatic GSs (A, B, C and D), IAA (E), and NPA (F). [Figure source: Textor and Gershenzon, 2009].

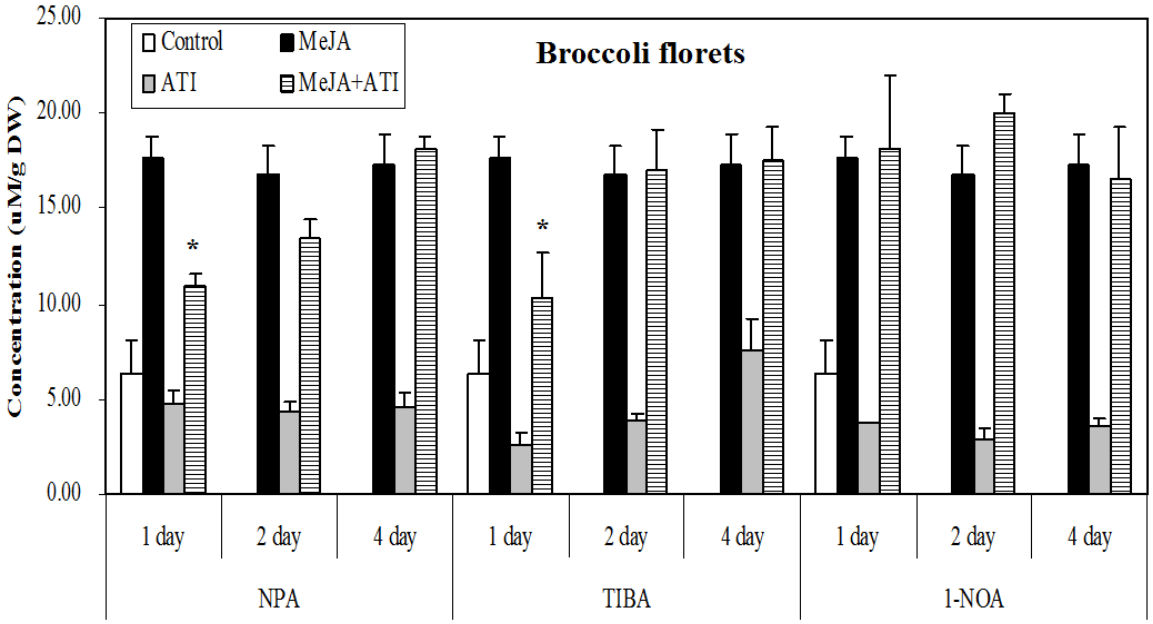


Figure 4.2. Changes in N-methoxy-3-indolylmethyl GS concentrations (μM/g DW) in floret tissues of 'Green Magic' broccoli 1, 2, and 4 days after control, MeJA, ATI (auxin transport inhibitor), and combined treatment of MeJA and ATI. Mean concentration of the plants subjected to the combined treatment of MeJA and ATI followed by a * indicate which were the significantly different from the concentrations of MeJA treated plants at $P \leqq 0.05$ using t-test.

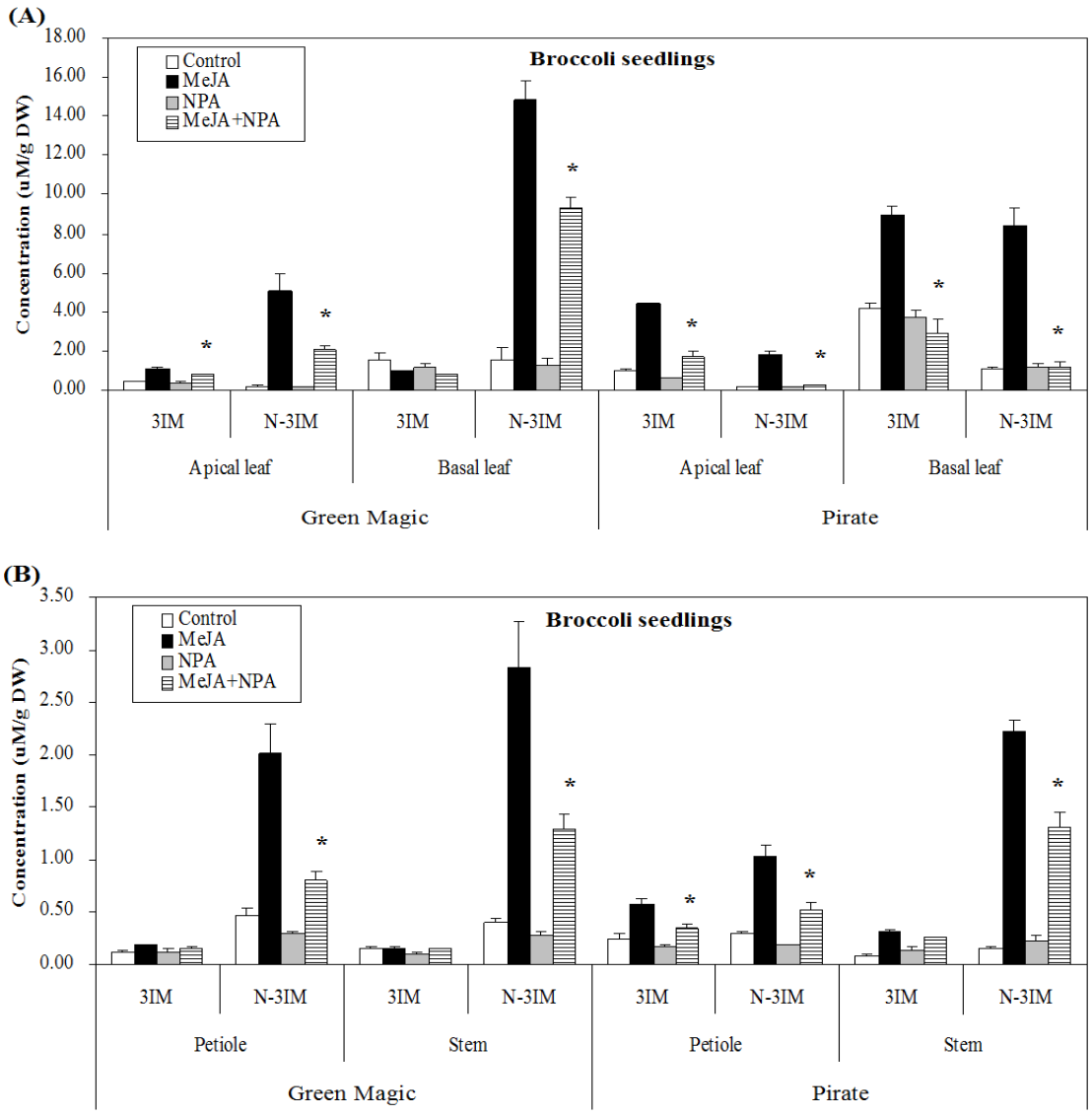


Figure 4.3. Changes in 3-indolymethyl GS and N-methoxy-3-indolylmethyl GS concentrations (μM/g DW) in apical and basal leaf tissues (A) and petiole and stem tissues (B) of the broccoli seedlings 2 days after control, MeJA, NPA, and combined treatment of MeJA and NPA, and in apical and basal leaf tissues of seedlings 2 days after control, basal leaf wounding, NPA, and combined treatment of wounding and NPA (C). Mean concentration of the plants subjected to the combined treatment of MeJA and NPA followed by a * indicate which were significantly different from the concentrations of plants treated only with MeJA at $P \leqq 0.05$ using the t-test. 3IM is an abbreviation of 3-indolymethyl GS, and N-3IM of N-methoxy-3-indolylmethyl GS.

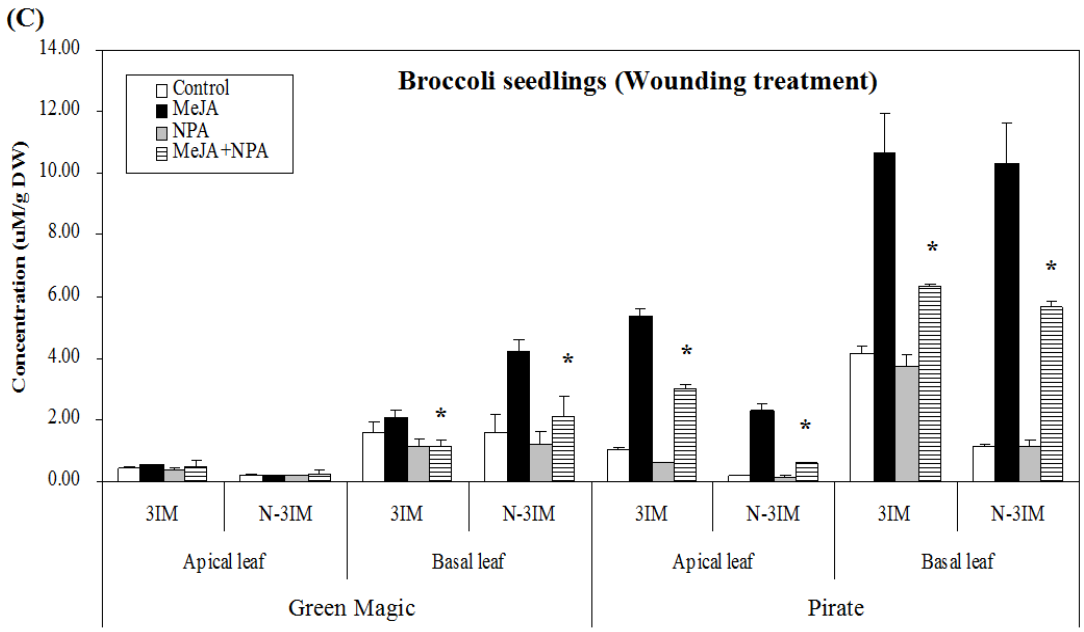


Figure 4.3. Continued.

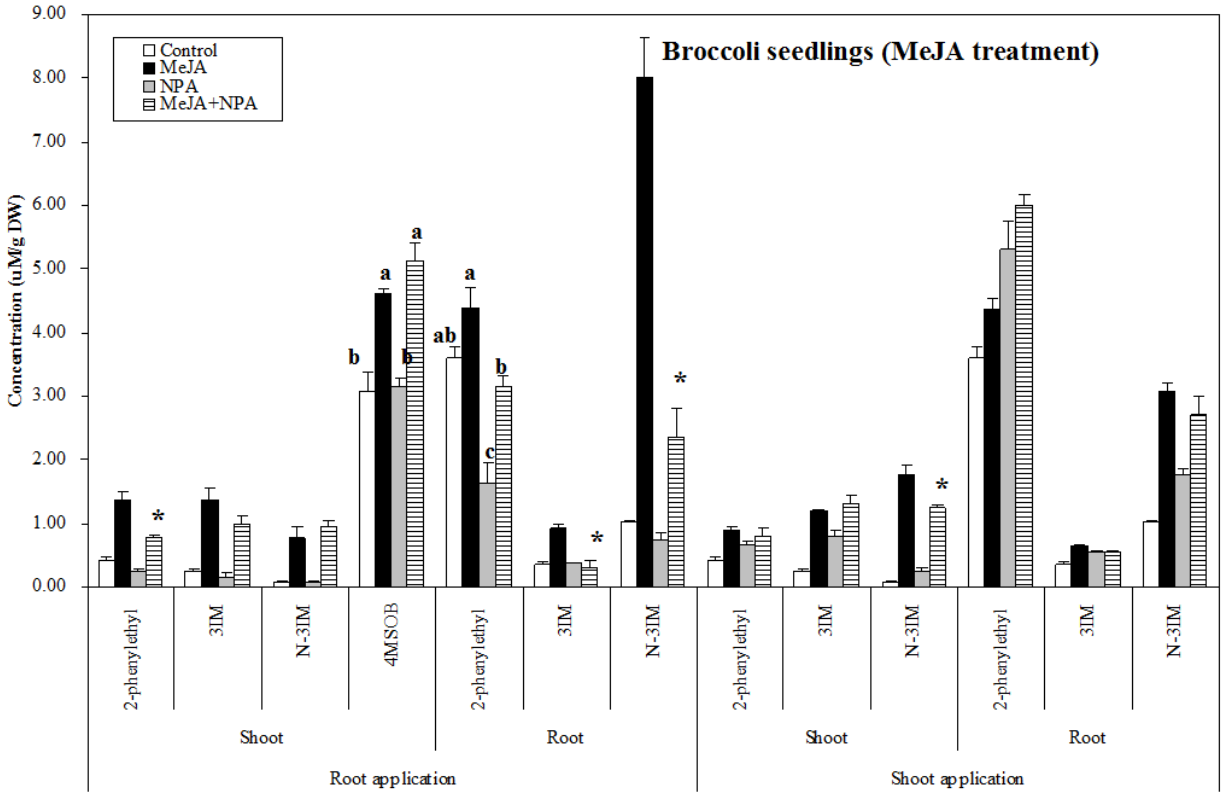


Figure 4.4. Effect of root and shoot application of control, MeJA, NPA, and combined solutions of MeJA and NPA on the concentration of 3-indolymethyl, N-methoxy-3-indolylmethyl and 2-phenylethyl GSs (μM/g DW) in 10-day old Green Magic broccoli sprouts. Mean concentration of the plants subjected to the combined treatment of MeJA and NPA followed by a * indicate which were significantly different from the concentrations of plant treated only with MeJA at p≦0.05 using the t-test. Means with a different letter are significantly different at P ≦0.05, using Fisher's LSD analysis. 3IM is an abbreviation of 3-indolymethyl GS, N-3IM of N-methoxy-3-indolylmethyl GS, and 4MSOB of 4-methylsulphinylbutyl GS.

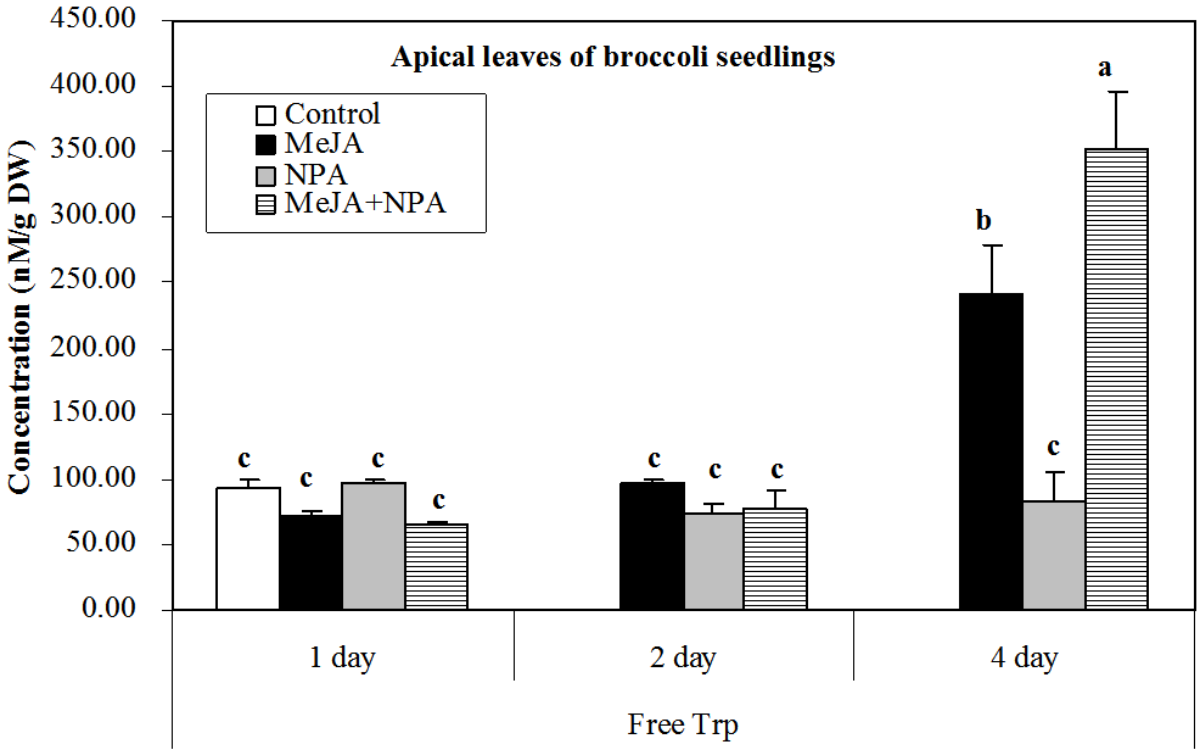


Figure 4.5. Free tryptophan levels in apical leaf tissues of broccoli seedlings 1, 2, and 4 days after control, MeJA, NPA, and combined treatment of MeJA and NPA.

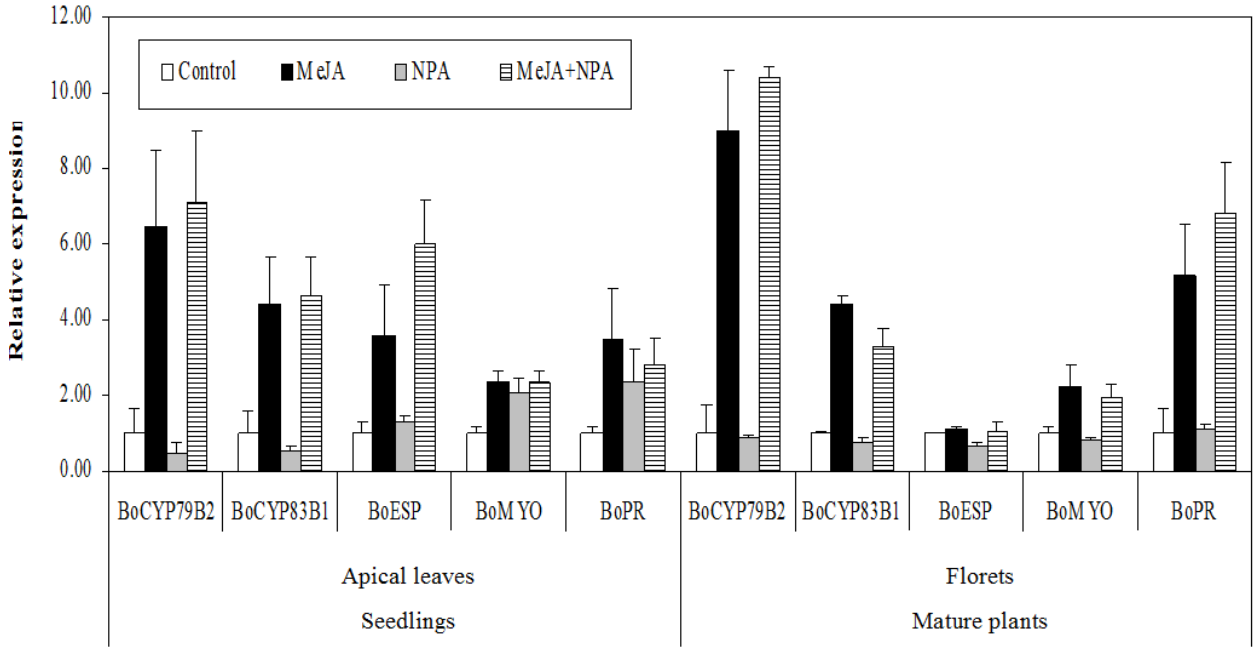


Figure 4.6. qRT-PCR analysis of transcript expression of genes involved in GS biosynthesis (*BoCYP79B2* and *BoCYP83B1*), and GS hydrolysis (*BoESP* and *BoMYO*), and plant defense (*BoPR*) in apical leaves of seedlings and florets of mature broccoli plants 1 day after control, MeJA, NPA, and combined treatment of MeJA and NPA. The transcript levels of genes were normalized with the *BoACT1* expression, and error bars represent standard error of means of triplicate biological replications.

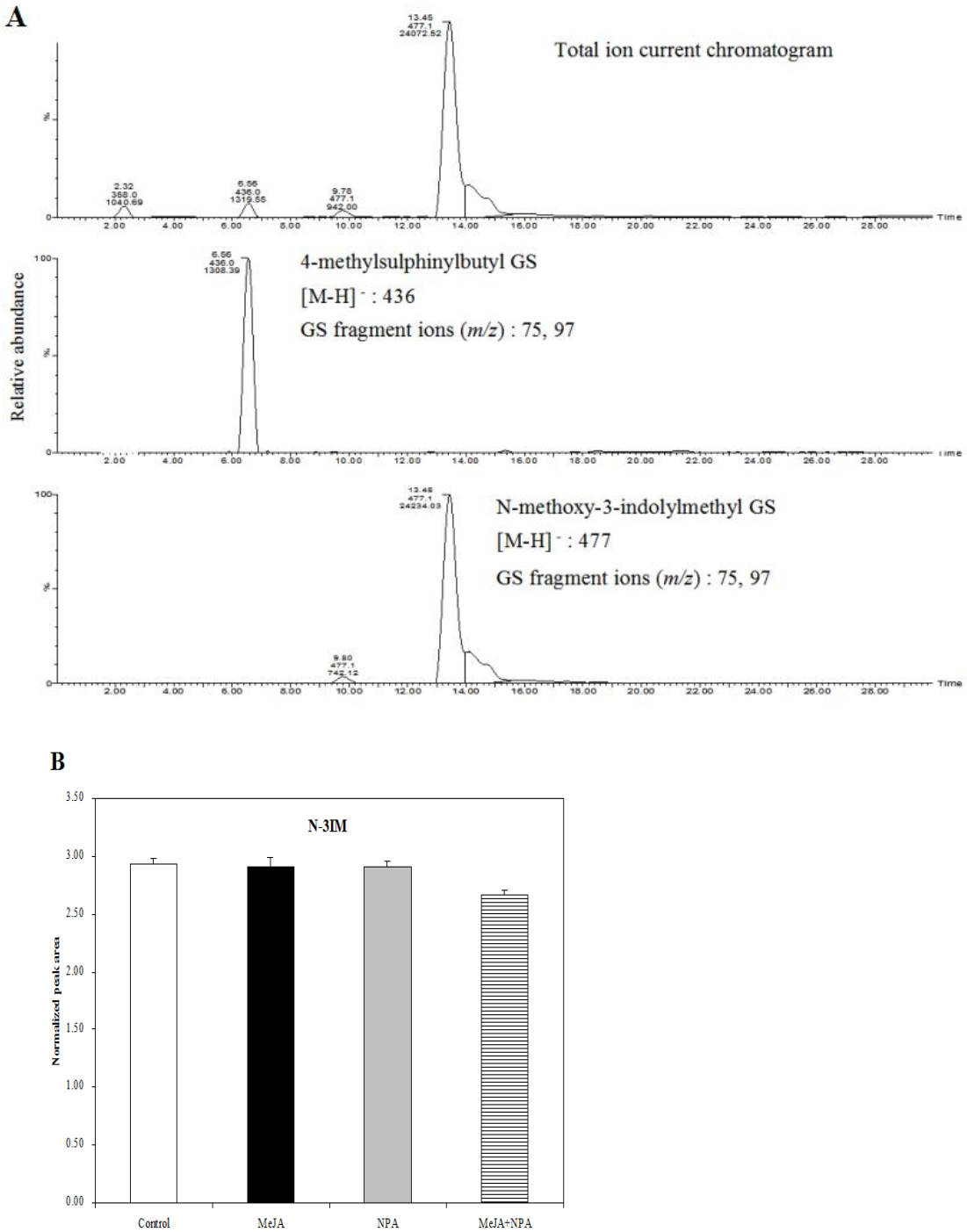
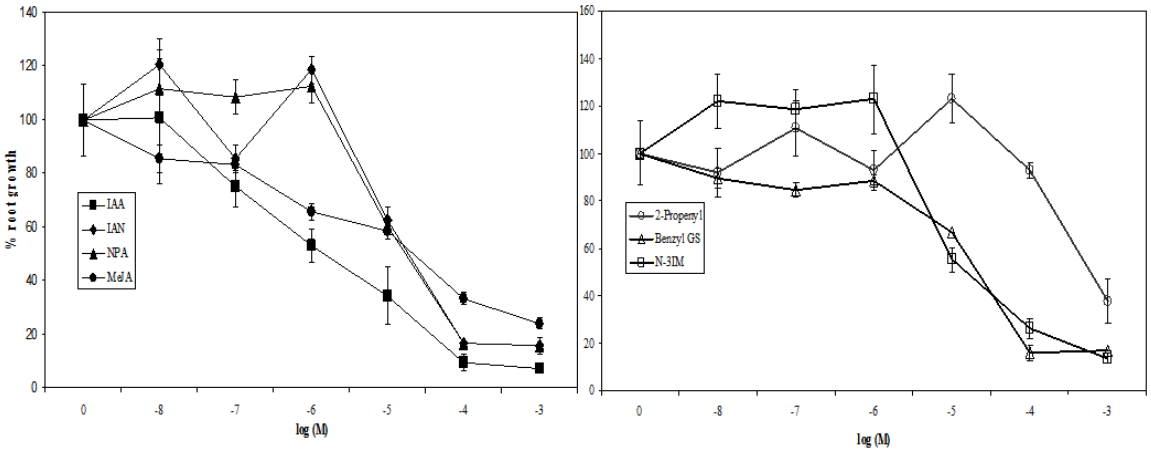
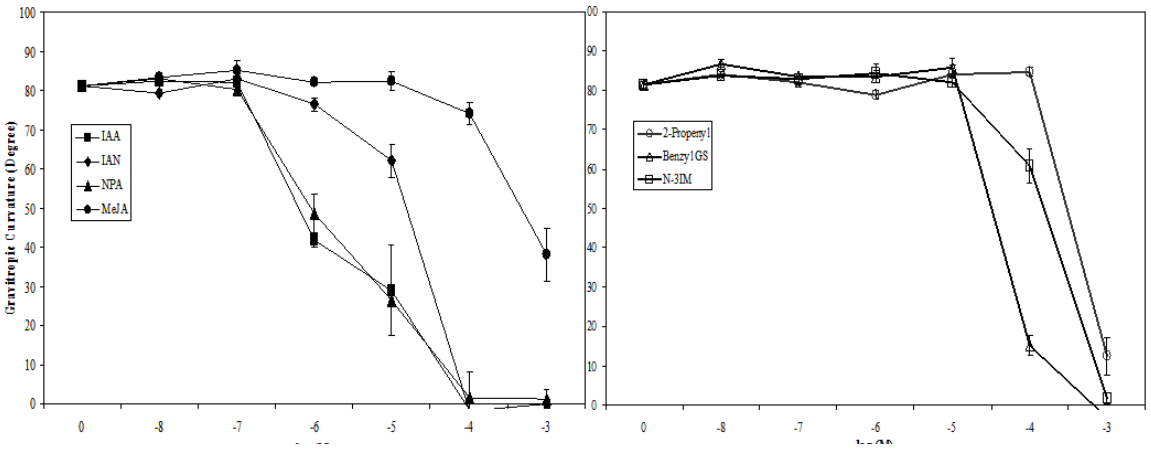


Figure 4.7. Mass chromatograms of GSs in broccoli phloem exudates detected by multiple reaction monitering (MRM) using negative ion LC-ESI/MS/MS (A). Relative abundance of N-methoxy-3-indolylmethyl GS (N-3IM) in phloem exudates extracted from the petiole of basal leaves 1 day after application of NPA, MeJA or the combined treatment of NPA with MeJA (B).

(A)



(B)



(C)

IC_{50} (concentration for 50% inhibition)	IAA	IAN	NPA	MeJA	2-Propenyl	Benzyl	N-3IM
Root growth	1 μM	30 μM	30 μM	40 μM	800 μM	35 μM	20 μM
Root gravitropic curvature	0.7 μM	20 μM	1 μM	700 μM	500 μM	50 μM	100 μM

Figure 4.8. Effect of exogeneous GS (2-propenyl, benzyl, and N-methoxy-3-indolylmethyl GS) treatments on *Arabidopsis* root growth (A) and gravitropism (B). IC50 was determined using the concentration of treatment which exhibited 50% inhibition on *Arabidopsis* root growth and gravitropic curvature (C). IAA, IAN, NPA and MeJA were also individually tested to compare the effect on root growing and gravitropism with those of GSs. Different concentrations ranging from 10 nM to 1 mM were used. N-3IM is an abbreviation of N-methoxy-3-indolylmethyl GS.

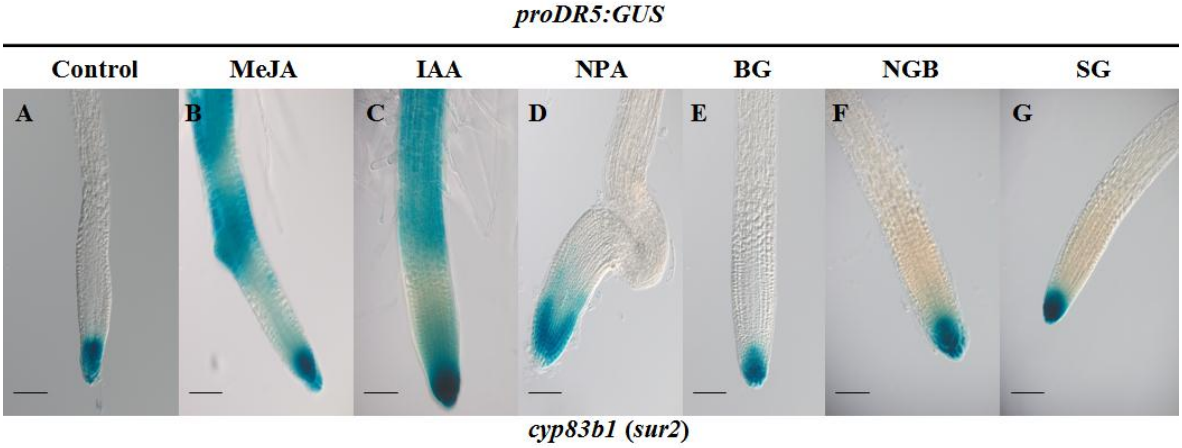


Figure 4.9. Effect of exogeneous GS treatments on *proDR5:GUS* expression in roots of *Arabidopsis* mutant *cyp83b1* (*sur2*). 4 days old seedlings were transferred onto medium containing control, 1 µM IAA, 20 µM NPA, 20 µM MeJA, 20 µM benzyl GS, 40 µM N-methoxy-3-indolylmethyl GS, and 500 µM 2-propenyl GS, and incubated for an additional 48 h. Bars = 100 µM.

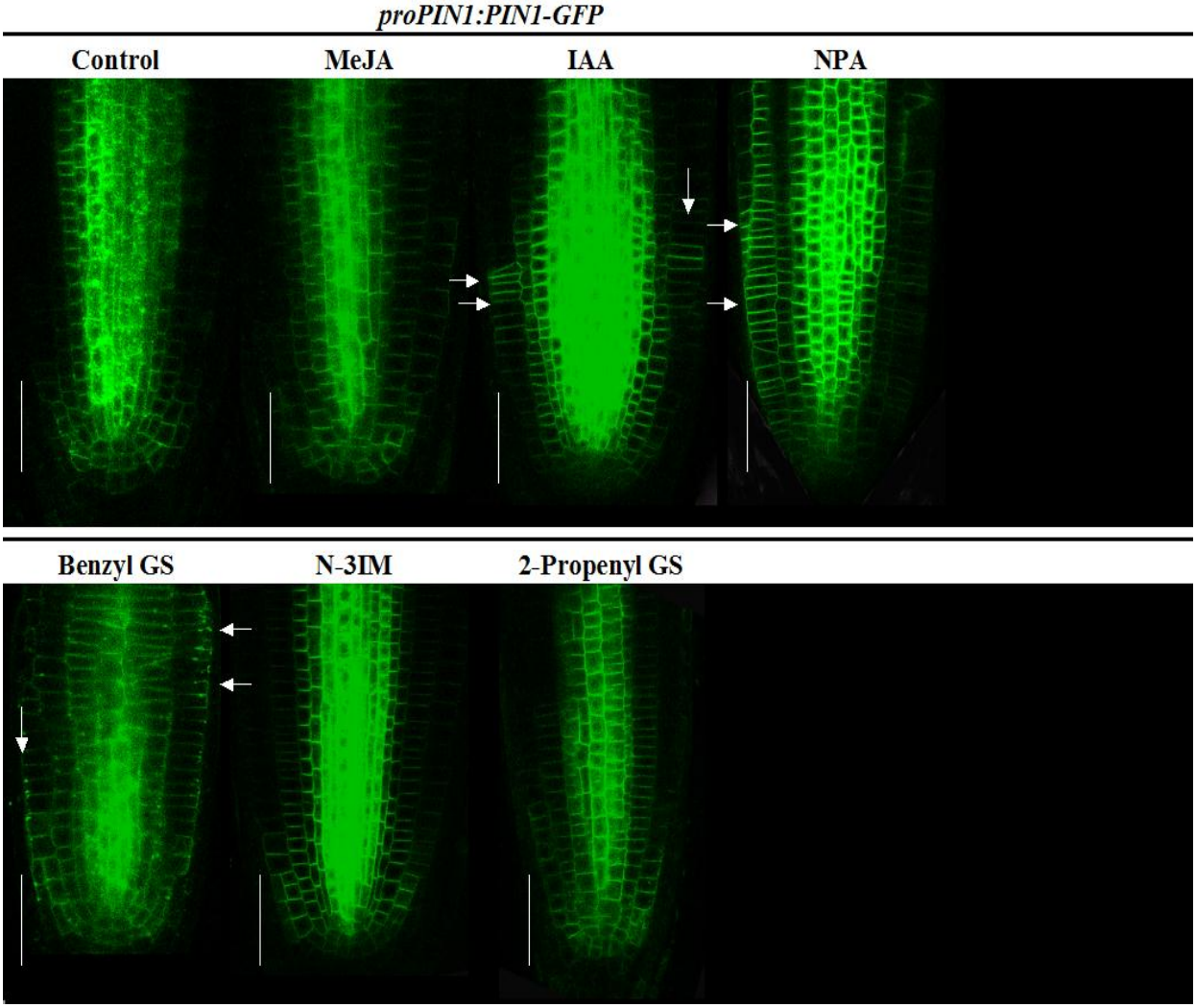


Figure 4.10. Effect of exogeneous GS treatments on PIN1 localization in root of *proPIN1:PIN1-GFP*. 4 days old seedlings were transferred onto the medium containing control, 1 μM IAA, 20 μM NPA, 20 μM MeJA, 100 μM benzyl GS, 500 μM N-methoxy-3-indolylmethyl GS, or 500 μM 2-propenyl GS, and incubated for an additional 48 h. GFP expression was visualized with a laser scanning confocal microscope. Images shown are representative of at least six independent experiments. Bars = 50 μM.

CHAPTER 5

Increasing levels of selenium and indolyl glucosinolates in broccoli florets to enhance health promoting bioactivity

5.1. Abstract

Broccoli (*Brassica oleracea* L. ssp. *Italica*) is a rich source of glucosinolates (GSs), phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Increasing dietary intake of the element selenium (Se) also has been shown to reduce the risk of cancer. Previous research reported that Se fertilization at high concentrations reduces the concentration of 4-methylsulphinyl GS (glucoraphanin), the GS precursor of sulforaphane, an isothiocynate known to upregulate genes in mammals associated with detoxification of dietary carcinogens.

This research reports on simultaneous enhancement of both Se and GS concentrations in broccoli floret tissue. Methyl jasmonate (MeJA) is a known elicitor, active in increasing concentrations of indolyl GSs and phenolic compounds in *Brassica* species. Five broccoli genotypes were subjected to root fertilization with solutions of Na_2SeO_4 and MeJA sprays to aerial portions of the plants in the greenhouse at floral initiation and 4 days prior to harvest of the head, respectively. Two different levels of Se fertilization (0.17 and 5.2 mM Na_2SeO_4) increased the concentration of Se in floret tissues across six genotypes from 3.0 (control) to 36.0 μg/g and to 520 μg/g, respectively. The low level of Se fertilization (0.17 mM of Na_2SeO_4) did not influence concentrations of all classes of GSs significantly, whereas the high level of Se treatment (5.2 mM Na_2SeO_4) decreased concentrations of 4-methylsulfinylbutyl, total aliphatic and total GSs by 34, 19 and 8%, respectively. The low level of Se fertilization with MeJA treatment displayed no significant changes in total aliphatic GS concentrations with 90% and

50% increases in indolyl and total GSs concentrations, respectively. These results suggest that Se- and GS-enriched broccoli with improved health-promoting properties can be generated by this combined treatment. Partitioning of the variance indicated that the existence of substantial variability in GS concentrations was primary attributed to differences in genotype response across different treatments. Results suggest that cultivar selection and breeding of broccoli can be employed to optimize the health-promoting effect of Se fertilization and MeJA treatments.

5.2. Introduction

Selenium (Se), a naturally occurring element found in many soils, is an essential micronutrient for animals and humans (Rayman, 2004; White and Broadley, 2005). Consumption of crops grown in Se-deficient soils suggests that 15% of the world's population have Se deficient diets (Rayman, 2002; White and Broadley, 2005). Se deficiency in humans is associated with cardiovascular disease, a weakened immune system, male infertility, and increased incidence of various cancers (Gupta and Gupta, 2002; Rayman, 2002; Finley, 2005; White et al., 2007). Dietary supplementation of 200 μg Se per day has been demonstrated to reduce cancer risk in humans (Clark et al., 1996), and consumption of Se-enriched broccoli also showed enhanced protection against colon and mammary cancer in laboratory animals compared to control broccoli alone (Finley et al., 2000; Davis et al., 2002; Finley et al., 2001). Se is also associated with the activity of several important antioxidant enzymes such as gluthathione peroxidase isozymes and thioredoxin reductase (Brown and Arthur, 2001). Se can not be added to food directly, since Se is not on the FDA's generally recognized as safe (GRAS) list (Finley et al., 2005). Se must be enriched naturally through Se fertilization leading to the accumulation and concentration of Se from food sources.

Brassica vegetables have received more attention due to their chemopreventive properties mainly attributed to glucosinolate (GS) degradation products. Among *brassica* vegetables, broccoli (*Brassica oleracea* L. ssp. *Italica*) is a rich source of GSs, phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Broccoli also has been shown to be a good source of several health promoting phytochemical compounds including carotenoids, tocopherols, and ascorbic acid (Ibrahim and Juvik, 2009). Broccoli has been considered as a candidate to supply both Se and GSs to the human diet (Finley et al., 2005; Keck and Finley, 2006). Although Se fertilization successfully increased Se accumulation in broccoli (Lee et al., 2005; Pedrero et al., 2008; Robbins et al., 2005; Sugihara et al., 2004), it also resulted in a dose-dependent decrease in GSs, especially in aliphatic GSs including 4-methylsulfinylbutyl GS (glucoraphanin), a parent GS of sulforaphane in broccoli floret and rapid-cycling *Brassica oleracea* (Charron et al., 2001; Toler et al., 2007). The isothiocyanate sulforaphane is highly potent at upregulating detoxification enzymes in cell culture and animal studies. Sulforaphane may also interfere with cancer promotion and progression via apoptosis (Zhang and Talalay, 1994; Gamet-Payrastre et al., 2000). Substantial reduction of GSs caused by Se fertilization is not conducive to the development of value-added vegetables with greater health-promoting capacity.

Methyl jasmonate (MeJA), a volatile methylester of jasmonic acid, has been demonstrated to induce the production of GS in *Brassica*, specifically of indolyl GSs (van Dam et al., 2003; Doughty et al., 1995; Robert P. Bodnaryk, 1994). The hydrolysis product of the 3-indolylmethyl GS (glucobrassicin), indole-3-carbinol, has proven successful in National Cancer Institute clinical trials against breast cancer (Telang et al., 1997) and respiratory papilloma (Rosen et al., 1998). Indole-3-carbinol has been reported to act as a chemoprotector by

bifunctional upregulation of Phase 1 and 2 detoxification enzymes in humans and was found to suppress the proliferation of various tumor cells in *in vitro* experiments (Wortelboer et al., 1992; reviewed in Aggarwal and Ichikawa, 2005). *N*-methoxyindole-3-cabinol, the hydrolysis product of *N*-methoxyindolyl-3-GS (neoglucobrassicin) shares similar anticarcinogenic activities with indole-3-carbinol (Neave et al., 2005; Stephensen et al., 2000). MeJA also has been known as an elicitor increasing the concentration of various phenolic compounds by activation of enzymes associated with the phenylpropanoid pathway (Kim et al., 2006; Kim et al., 2007).

Identification or development of genotypes with enhanced and stable levels of Se and GSs are particularly important to provide a value-added inducement for increased broccoli consumption with superior health promotion effects. The feasibility of Se fertilization and MeJA treatment for improvement in Se and GS concentrations can be evaluated by partitioning of the phenotypic variability into its component sources [genotype, treatment and genotype × treatment (G×T) interaction]. If a high proportion of the phenotypic variance for a specific trait is described by treatment, the corresponding treatment may be feasible to improve trait performance. If the dominant proportion of phenotypic variance is due to genotypic differences, this indicates the feasibility of genetic manipulation to improve the trait. The proportion of phenotypic variance described by G×T interaction can be used to estimate a breeding potential to create genotypes optimized for specific treatments.

The aim of this study is to enhance both Se and GS concentration in broccoli florets through the combined application of Se fertilization and MeJA treatment. We have investigated changes in Se and GS concentrations in broccoli floret tissue among 6 different broccoli cultivars after Se fertilization, MeJA treatment, or a combination of these treatments. The same treatments were applied to seedlings of one genotype to compare different responses in GS concentration

between leaf tissue of seedlings and the florets of mature plants. Total sulfur and Se concentrations in the leaf tissue of seedling and floret of mature plant were determined in the selected genotypes to estimate effects of treatments and interaction between these elements and GS concentrations. Phenotypic variation in GS concentration among broccoli tissue samples was evaluated and partitioned into the genetic, treatment, and G×T interaction components of variance.

5.3. Materials and methods

5.3.1. Plant material

The first experiment was conducted to analyze the variation in Se accumulation occurring in the florets of six broccoli genotypes after Se fertilization. The florets of six broccoli genotypes, including two doubled haploid genotypes, 'VI-158' and 'SU003' (courtesy of Dr. Mark Farnham at the USDA Vegetable Improvement Center in Charleston, SC), and four commercial hybrid cultivars, 'Brigadier' and 'Pirate' (Asgrow Seed Co.), and 'Sultan' and 'Green Magic' (Sakata Seed Co.) were subjected to two different levels of Se fertilization (0.17 or 5.2 mM Na_2SeO_4 solution) with controls.

The effects of MeJA treatment and potential interactions between MeJA and Se treatment on GS concentrations were studied in the second and third experiments. As a second experiment, florets of five genotypes excluding cultivar 'Pirate' from the six genotypes in the first experiment were subjected to the MeJA treatment or a combined treatment of MeJA with a high level of Se fertilization (5.2 mM Na_2SeO_4). In the third experiment, two cultivars 'Sultan' and 'VI-158' were selected based on the results of the first and second experiments, and subjected to the combined treatment of MeJA and both low and high level of Se fertilization (0.17 and 5.2 mM Na_2SeO_4 solution).

In a fourth experiment, the effect of MeJA, high levels of Se fertilization or combination of both treatments on GS concentrations was investigated in broccoli seedlings to compare with the effects of these treatments on GS accumulation in broccoli floret tissues. Six week old seedlings of the 'Green Magic' were subjected to the high level of Se fertilization, MeJA treatment or the combination of both treatments.

Seeds of each broccoli genotype were germinated in small pots filled with sunshine® LC1 professional soil mix. For the first, second and third experiments, four-week old seedlings were transplanted into 22.8 cm pots and grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25℃/15℃ and 14-h/10-h day/night temperature regime and with supplemental light. Three weeks after transplanting, plants were fertilized with a solution of 20N-20P-20K twice a week. Experimental design was a randomized complete block with three replicates of 3 plants spaced 30 cm apart with 30 cm between rows on greenhouse benches. In the fourth experiment, four week old seedlings were transplanted into 5 cm pots and grown for two weeks under the same greenhouse conditions as described above. Experimental design was also a randomized complete block with three replicates of 3 seedling plants.

5.3.2. Se fertilization and MeJA treatment

In the first experiment, two different levels of Se fertilization were applied as soil drenches to the broccoli plants at floral initiation stage as described by Finley et al. (2000). Briefly, when plants initiate floral primordial, 10 mL of 0.17 and 5.2 mM Na_2SeO_4 solution (low and high level of Se fertilization, respectively) were applied into each plant container 2 times/wk until plants formed immature inflorescences approximately 2.5 cm in diameter, and then 20 mL of each solution was added 2 times/wk until heads were fully formed and at market harvest maturity.

In the second and third experiments, broccoli floret tissues were subjected to the MeJA treatment four days prior to commercial harvest maturity. All aerial plant parts including floret, stem and leaf tissues were sprayed with 250 µM MeJA (Sigma-aldrich, St. Louis, MO) containing 0.1% Triton X-100 (Sigma-aldrich, St. Louis, MO) until plant surfaces were fully saturated with MeJA solution. Control plants were sprayed with water containing 0.1% Triton X-100. Plants were spray treated in isolation and completely dried prior to being placed back onto greenhouse benches. Combination treatments of Se and MeJA were conducted by spraying 250 µM MeJA solution onto plants fertilized with high and low levels of Se (5.2 or 0.17 mM Na_2SeO_4 solution) for the second and third experiment, respectively. Broccoli heads was cut below the last branch of the immature inflorescence to exclude the thick stem portion. Equal subsamples of the edible portions of the three heads were combined, frozen in liquid nitrogen, lyophilized, ground into powder and stored at -20℃ until analysis.

In the fourth experiment, 5 mL of 5.2 mM Na_2SeO_4 solution was applied to each four week old seedling, 3 times/wk for two weeks. 250 µM MeJA or control solution was sprayed onto all aerial parts of seedlings 1, 2 and 4 days prior to tissue harvest. Apical (second and third leaf node from the apical meristem) and basal (4^{th}-7^{th} leaf node) leaves were harvested and combined in each of the three replicates, and frozen in liquid nitrogen, lyophilized, ground into powder and stored at -20℃ until analysis.

5.3.3. Glucosinolate analysis

GSs in lyophilized floret tissue were extracted and analyzed by high-performance liquid chromatography using a reverse phase C^{18} column as described by Wathelet et al. (1991) and Brown et al. (2002) with some modifications. GSs were desulfated with sulfatase solution (Sigma-Aldrich, St. Louis, MO) in columns containing DEAE Sephadex A-25 resin (Sigma-

Aldrich, St. Louis, MO) and eluted from the column with deionized distilled water. Desulfo-GSs were separated on a HPLC system consisting of a DIONEX GP40 gradient pump, AD20 UV absorbance detector (Sunnyvale, CA) set at 229 nm wavelength, auto-sampler, all-guard ™ cartridge guard column (Alltech, Lexington, Kentucky), and a Lichosphere 100 RP-18 column (Grace Co. Deerfield, IL). Desulfo-GSs were eluted off the column in 45 min with a linear gradient of 0% to 20% acetonitrile in water at a flow rate of 1.0 mL/min. The type and amount of GSs in each sample were calculated in comparison to certified glucosinolate levels in a standard rapeseed reference material (BCR 367, Commission of the European Community Bureau of References, Brussels, Belgium). GSs were quantified with benzylglucosinolate (POS Pilot Plant Corp, Saskatoon, SK, Canada) as an internal standard using UV response factors for different types of GSs determined by Wathelet et al (2001). The recovery of GSs from the samples under this procedure was estimated at 95 to 97 % (Brown et al., 2002). The identification of desulfo-GS profiles was validated by LC/MS. The molecular ion and fragmentation patterns of individual desulfo-GS were matched with the literature for validation (Barbieri et al., 2008).

5.3.4. Determination of Selenium and sulfur concentration

Analysis of tissue Se and sulfur concentrations was performed by the Division of Agriculture and Natural Resources (ANR) Analytical Laboratory, University of California at Davis. Total Se was analyzed by nitric acid-perchloric acid-sulfuric acid digestion and determination by Vapor Generation Inductively-Coupled Plasma Emission Spectrometry (VG-ICP) (Tracy and Moeller, 1990). Total Sulfur content was determined through a nitric acid-hydrogen peroxide microwave digestion and determination by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Sah and Miller, 1992).

5.3.5. Statistical analysis

GS, total Se and Sulfur concentrations obtained from the broccoli plants treated with control, Se fertilizer, MeJA or combination of Se fertilizer and MeJA were analyzed by the GLM procedure and Fisher's LSD tests using the SAS statistical analysis package (SAS version 8.0).

Analysis of variance using PROC GLM (SAS Institute, 2000) was performed on the data based upon the linear model: $x_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \tau_{k(j)} + e_{ijk}$, where x_{ijk} is the kth replicate of the phenotypic value of the ith genotype in treatment j, μ is the overall mean, α_i is the fixed effect of genotype i, β_j is the fixed effect of treatment j, $(\alpha\beta)_{ij}$ is the fixed interaction effect of genotype i in treatment j, $\tau_{k(j)}$ is the nested effect of the kth block within the jth treatment, and e_{ijk} is the experimental error associated with x_{ijk}. F tests at $P < 0.05$ were used to determine the significance of the individual variance components.

5.4. Results

5.4.1. Effect of selenium fertilization on Se and GS accumulations in broccoli florets

The total Se concentrations determined in florets of the 6 broccoli genotypes with or without Se fertilization are given in Table 5.1 and Figure 5.1. Two different levels of Se fertilization resulted in a dose-dependent increase of Se concentration in floret tissues of the 6 genotypes ranging from 0.0 to 13.0 μg of Se/g of dry tissue for the control treatment, from 17.7 to 51.0 μg/g under the low level Se fertilization (0.17 mM Na_2SeO_4), and from 193 to 745 μg/g for high level Se fertilization (5.2 mM Na_2SeO_4). Se accumulations in floret tissues under the high level of Se fertilization were significantly different among the genotypes (Figure 5.1). Cultivar 'Sultan', 'Green Magic', and 'SU003' had higher levels of Se in floret tissues than the other genotypes. The overall increase of Se concentrations averaged across the 6 genotypes

ranged from 3.0 (control) to 36.0 µg/g (low level of Se fertilization) and to 520 µg/g (high level of Se fertilization).

The effect of two different levels of Se fertilization on GS accumulation in floret tissues varied among 6 broccoli genotypes (Table 5.2). The high level of Se fertilization decreased the concentration of 4-methylsulphinyl GS (glucoraphanin) and total aliphatic GSs in 3 out of 6 genotypes. No significant reduction in 4-methylsulphinyl GS (glucoraphanin) and total aliphatic GS concentrations were observed in cultivar 'Brigidier' and 'Pirate' which showed relatively less Se accumulation compared to other genotypes under high Se fertilization and relatively low control levels of 4-methylsulphinyl GS. Indolyl and aromatic GSs did not show a consistent response to Se fertilizations across the genotypes suggesting that there is little or no influence of Se fertilization on accumulation of these GSs in florets. Accumulation of 4-methylsulphinyl GS in broccoli florets varied both in control and Se treated plants among the 6 cultivars (Table 5.1). Higher concentrations of 4-methylsulphinyl GS were observed in control treatments of 'VI158' and 'Green Magic', and these cultivars exhibited a more dramatic reduction in 4-methylsulphinyl GS concentrations (50% and 53% reduction, respectively) in plants treated with high level of Se fertilization.

5.4.2. MeJA treatment increases indolyl GS accumulation in floret tissues

Changes in GS profiles after MeJA or the combined treatment of MeJA with high levels of Se in florets of 5 broccoli genotypes is shown in Table 5.2. The concentrations of indolyl and aromatic GSs significantly increased in florets of 4 out of 5 cultivars after MeJA treatment, and these increases made the total GS concentrations greater than untreated plants in all genotypes. Accumulation of two indolyl GSs, 3-indolylmethyl and N-methoxy-3-indolylmethyl GSs in florets demonstrated genotype-dependent responses for MeJA treatment (Figure 5.2). N-

methoxy-3-indolylmethyl GS concentrations in two genotypes ‘Green Magic’ and ‘SU003’showed the most dramatic increases (ranging from 2.8- to 4.3-fold over controls) with the cultivar ‘Sultan’ providing the highest dose of this compound (18.4 μM/g of dry weight) with MeJA treatment. Dominant accumulation of N-methoxy-3-indolylmethyl GS after MeJA treatment was observed in all genotypes except ‘VI-158’ which showed a 2.6 fold increase of 3-indolylmethyl GS and was the only line to display greater 3-indolylmethyl GS than N-methoxy-3-indolylmethyl GS concentrations after MeJA treatment. Combined treatment of MeJA with the high level of Se fertilization decreased total aliphatic GS concentrations comparable to the sole treatment of high Se, indicating no positive effect of combined treatments on aliphatic GS accumulation.

Increases in total GSs in plants grown under Se fertilization were tested in florets of cultivar ‘Sultan’ and ‘VI-158’ through the combination treatment of the low level fertilization of Se with MeJA spray (Table 5.2). Total aliphatic GS concentrations were not significantly changed under the combined treatment similar to results obtained from the plants subjected to the low level of Se fertilization alone. Combined treatments of low levels of Se with MeJA increased total GS levels by 24% and 28% in ‘Sultan’ and ‘VI-158’, respectively, compared with the combined treatment of high levels of Se fertilization and MeJA. This indicates that MeJA-mediated increase in indolyl GSs also can be inhibited by Se fertilization, depending on the concentration of Se fertilization and genotype-specific responses to Se fertilization.

5.4.3. Variation in GS concentrations among the 5 broccoli genotypes

Overall changes of individual GS concentrations in floret tissues across the 5 genotypes under the two different levels of Se fertilization (0.17 and 5.2 mM Na_2SeO_4 solution, respectively), MeJA treatment and combination of both treatments are displayed in Table 5.3.

The low level of Se fertilization did not influence concentrations of all classes of GSs significantly, whereas the high level of Se treatment decreased concentrations of 4-methylsulphinyl, total aliphatic and total GSs by 34, 19, and 8%, respectively. Concentrations in the aromatic GS, 2-phenylethyl GS, and two indolyl GSs, 3-indolylmethyl, and N-methoxy-3-indolylmethyl GS, were not significantly changed by two different levels of Se fertilization, indicating no negative effect of Se fertilization (0.17 and 5.2 mM Na_2SeO_4 solution) on aromatic and indolyl GS concentrations in florets across the 5 genotypes. MeJA treatment alone increased concentrations of 2-phenylethyl, 3-indolylmethyl, and N-methoxy-3-indolylmethyl GS significantly, with average total indolyl GS and total GS concentrations increased by 2.0 and 1.5 fold, respectively, over control treatments. The combination treatment of both MeJA and high level of Se resulted in a 36% reduction of total aliphatic GS concentrations compared to the control treatment, but the increase in indolyl GS concentrations derived by MeJA treatment compensated for the loss of aliphatic GSs and consequently made total GS levels 4 and 12% higher than control and the high level of Se treatment, respectively. Under the combined treatments, the concentrations of 2-phenylethyl and 3-indolylmethyl were not significantly increased compared to the control treatments, suggesting that the MeJA-mediated increase in these compounds was inhibited under high levels of Se fertilization. The concentration of N-methoxy-3-indolylmethyl GS significantly increased under the combined treatments, but were lower than in MeJA treatment alone. The increase in N-methoxy-3-indolylmethyl GS concentrations mediated by MeJA treatment was also inhibited by high levels of Se fertilization, but was less affected than 2-phenylethyl and 3-indolylmethyl GSs. The increase in N-methoxy-3-indolylmethyl GS concentration led to increases in total GS concentration in florets subjected to the combination treatments (Table 5.3).

5.4.4. Effect of Se fertilization on GS accumulation in broccoli seedlings

Previous research analyzing the effect of Se fertilization on GS accumulation have shown different responses between the shoot tissues of rapid-cycling *Brassica oleracea* and floret tissues of broccoli (Charron et al., 2001; Robbins et al., 2005). Greater reduction in total GSs (about 67% reduction) was found in shoot tissues of rapid-cycling *Brassica oleracea* compared to floret tissues of broccoli (about 27 % reduction in total GSs) after Se fertilization, while the respective tissues accumulated comparable Se levels. To analyze the effect of Se fertilization on GS concentrations in different tissues and developmental stages of broccoli, six week old seedlings of 'Green Magic' were subjected to the high level of Se fertilization (5.2 mM Na_2SeO_4), MeJA treatment or the combined treatment. Similar to floret tissues, MeJA increased concentrations of indolyl, aromatic and total GSs in both apical and basal leaves of six week old broccoli seedlings. However, both the high level of Se fertilization and combined treatment of MeJA with the high level of Se fertilization reduced aliphatic and indolyl GS accumulation in both apical and basal leaves (Figure 5.3). Total GS concentration decreased by 63% and 72% in apical leaves of seedlings under high Se and the combined treatment, respectively, with 50% and 33% reduction of total GSs for the same treatments in basal leaves. The high level of Se fertilization reduced GS accumulation, and completely inhibited MeJA–mediated indolyl GS accumulations in both apical and basal leaves of seedlings. In contrast floret tissues of the same genotype subjected to the same treatments had comparable levels of total indolyl-GS and showed only a 9% decrease in total GS concentrations under the combined treatment (Table 5.2).

5.4.5. Changes in sulfur and Se concentration caused by Se fertilization, MeJA treatment or the combined treatment of Se with MeJA

Total Se and sulfur concentrations in florets and basal leaves of seedlings under high Se fertilization , MeJA application or combination treatment are shown in Figure 5.4. The high Se fertilization treatment did not cause any significant changes in sulfur concentrations in the florets of the 'Green Magic' and 'Sultan', whereas high Se and the combined treatment in basal leaves of 'Green Magic' decreased sulfur concentration' by 36 % and 33 %, respectively, compared to the control treatment in basal leaves of Green Magic seedlings. This suggests the competitive inhibition of sulfur uptake with Se fertilization in seedlings. MeJA increased sulfur concentrations in florets and in basal leaves of seedlings, but only the florets of 'Green Magic' showed significant differences in sulfur concentration compared with controls (Figure 5.4).

Se fertilization resulted in an increase in Se concentrations in basal leaves of seedlings up to 1454 μg/g (high level of Se fertilization). This level of Se was much greater than that found in florets of same genotype which ranged up to 576 μg/g (high level of Se fertilization) (Figure 5.4). The Se concentration determined in the basal leaves of seedlings did not show significant differences between the high level of Se fertilization and the combination treatment of MeJA and high level of Se fertilization, indicating no interaction between treatments. However, the Se levels detected in florets displayed significant differences in Se concentration between the high level of Se fertilization and the combination treatment of Se with MeJA in both 'Green Magic' and 'Sultan' (Figure 5.4). Increased sulfur concentration in florets of 'Green Magic' subjected to MeJA treatment may be due to increased sulfur uptake stimulated by this treatment, which may competitively inhibit the Se uptake from the soil.

5.4.6. Genotype and treatment variation in GS concentrations

Analysis of variance was applied to partition the total phenotypic variation of the GSs into components associated with genotype, treatment, and G×T interaction (Table 5.4). Percentage of the total variation for aliphatic and indolyl GS concentrations varied depending on different treatment methods. In the high level of Se fertilization, most of the variation (78.2-86.0% of the total variation described by the model) in indolyl GSs concentrations was described by the genotypic effects, while the variation of total aliphatic GSs were primarly associated with the Se treatment and Genotype × Se treatment interaction (18.2 % and 50.3 %, respectively). In contrast, the genotypic effects described most of variation for total aliphatic GSs (89.7 %) in the MeJA treatment, where the total indolyl GSs variability was primarly attributed to MeJA treatment effect (43.0 %). The combined treatment of high level of Se with MeJA spray increased the percentage of variation caused by G×T interactions in all classes of GSs significantly, suggesting that the combined treatment impacts the stability of both aliphatic and indolyl GS concentrations among individual genotypes.

5.5. Discussion

Our experimental data demonstrates that Se enhancement in broccoli florets can be achieved through Se fertilization. Variation in Se accumulation among the 6 broccoli genotypes was observed in florets of plants subjected to Se fertilization (Figure 5.1). This result suggests that selection and breeding can be performed to develop germplasm with enhanced capacity of Se acquisition and accumulation in broccoli. The two different levels of Se fertilization (0.17 and 5.2 mM Na_2SeO_4) increased the concentration of Se in floret tissues across the six genotypes from 3.0 (control) to 36.0 μg/g and to 520 μg/g, respectively. The current recommended dietary allowance (RDA) for human in the USA is 55 μg (0.7 μmol) of Se per day (Rayman, 2004;

White and Broadley, 2005). Epidemiological studies have indicated that supplementation with 200 μg Se per day to healthy men and women substantially decreases total cancer incidence and mortality (Duffield-Lillico et al., 2002). Accordingly, a daily consumption of approximately 5.5 g dry weight (approximately 50 g wet weight) of broccoli florets produced under the low level of Se fertilization could provide a level of Se to satisfy the RDA and dietary supplementation of 200 μg Se. On a cautionary note consumption of excess selenium may cause Se toxicity in humans although only a portion of the Se in foods is uptaken in the human digestive process. The current National Research Council (NAS) has set the recommended upper intake level for Se at 400 μg per day for adults (MacFarquhar et al., 2010), which would equate to the amount of Se found in approximately 100 g wet weight of broccoli floret tissue from the low level Se treatment.

Broccoli is a rich source of GSs, phytochemicals that are hydrolyzed into isothiocyanates with known human anti-carcinogenic bioactivity. Increasing dietary intake of the element Se also has been shown to reduce the risk of cancer. Simultaneous enhancement of both Se and GS levels in broccoli floret tissue would produce synergetic health promoting effects among consumers. However, previous research have demonstrated that the Se fertilization at high concentrations reduces the accumulation of 4-methylsulphinyl GS (glucoraphanin), the GS precursor of sulforaphane, an isothiocynate, as well as phenolic acids (Finley et al., 2005). In our experiments, the effect of Se fertilization on GS accumulations varied depending on genotypes, developmental stages of plant and the level of Se fertilization. The high level of Se fertilization (5.2 mM Na_2SeO_4) decreased in 4-methylsulphinyl GS and total aliphatic GS concentrations across the 6 broccoli genotypes. The low level Se fertilization treatment (0.17 mM Na_2SeO_4) did not cause any significant changes in both aliphatic and indolyl GSs. This result is consistent with the previous research reported by Robbins et al (2005). They reported that two different levels of

Se fertilization resulted in a dose-dependent decrease in aliphatic and total GS concentrations with no significant effect on indolyl GS accumulation in broccoli florets of cultivar 'Majestic' (Robbins et al., 2005).

Aliphatic GSs, specifically 4-methylsulphinyl GS sensitivity to Se fertilization may be a consequence of reduction in plant cysteine and methionine biosynthesis caused by the competitive biosynthesis of Se-cysteine and Se-methionine. GSs contain at least two sulfur atoms, one originating from cysteine and the other from phosphoadenosine phosphosulfate. Methionine-derived aliphatic GSs contain an additional reduced sulfur atom (Rausch and Wachter, 2005). It can be assumed that methionine-derived aliphatic GS biosynthesis may be more strongly sulfur or sulfur-containing amino acids-dependent compared to indolyl glucosinolates derived from tryptophan (Schonhof et al., 2007). Decreases in aliphatic GS concentrations with no significant change in sulfur concentration under the high level of Se treatment in broccoli florets also suggest that the reduction in aliphatic GSs may be influenced by decreased levels of sulfur-containing amino acids such as cysteine and methionine rather than by overall sulfur content in the plant. Increased sulfur uptake with reduced GS concentrations under a Se fertilization treatment was also observed in rapid-cycling *Brassica oleracea* (Charron et al., 2001; Toler et al., 2007).

MeJA treatment increased the amount of indolyl GSs through the highly induced expression of the corresponding Trp-metabolizing genes *CYP79B2* and *CYP79B3* catalyzing the conversion of tryptophan to indole-3-acetaldoxime and conversion of aldoximes to thiohydroximic acids, respectively (Halkier and Gershenzon, 2006). MeJA-mediated increases in indolyl and aromatic GSs in floret tissue was a consistent response across the broccoli lines tested in our experiment. Simultaneous increases of Se and indolyl GSs can be achieved in

broccoli florets through the combination treatment of MeJA with Se fertilization. Similar to sulforaphane, phenylethyl isothiocyanate derived from 2-phenylethyl GS (gluconasturtiin) and *N*-methoxyindole-3-carbinol, the hydrolysis product of N-methoxy-3-indolylmethyl GS, also have human anti-carcinogenic bioactivity (Staack et al., 1998; Neave et al., 2005). Enhanced concentrations of Se and indolyl GSs in broccoli florets mediated by the combination of Se and MeJA treatment may provide beneficial effects for human health promotion.

GS profiling data obtained from broccoli seedlings and florets after Se, MeJA or the combined treatment can provide information to estimate net GS biosynthesis capability in different developmental stages of plants responding to limited sulfur conditions caused by competitive uptake and assimilation between sulfur and Se. The high level Se treatments depressed accumulation of all classes of GSs and completely inhibited MeJA-mediated indolyl GS biosynthetic upregulation in broccoli seedlings. These results are in contrast to those observations in floret tissue where MeJA increased indolyl GS concentrations in the high Se treatment. The different response between seedlings and mature plants may be due to the differences in size of available pools of GS amino acid precursors and sulfur required for *de novo* GS biosynthesis. Increased accumulation of indolyl GSs in floret tissues in the combined treatment may be from translocation of GSs or GS precursors from sink tissues such as leaves, roots or stems to the floret. Significant increases in indolyl GS concentrations were detected in leaf and stem tissues in mature broccoli plants after MeJA treatment suggesting translocation of GS from source to sink tissues (Chapter 2).

The existence of substantial variability in GS concentrations attributed to differences in the genotypes across different treatments suggest that genetic manipulation can be conducted to increase GS concentrations under the Se fertilization, MeJA, or the combined treatments. The

high proportion of the phenotypic variation for aliphatic GSs was described by Genotype × Se treatment interactions, suggests it would be feasible to select and develop germplasm with optimized Se uptake. In a breeding program to develop broccoli with enhanced health promotion, attention should be given to identify germplasm where GS biosynthesis (particularly 4-methylsulphinyl GS) is not as sensitive to Se fertilization and uptake. Further studies need to be conducted in the field to test these observations generated from greenhouse studies.

Table 5.1. Effect of different levels of Se fertilization on the concentration of Se (± SE; μg/g DW) and GSs (± SE; μM/g DW) in floret tissues of the 6 broccoli genotypes.

Genotype	Na_2SeO_4 (mM) treated	Total Se	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
						4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Green Magic	0	13.0 ± 10 b [y]	9.5 ± 1.0 a	9.5 ± 2.3 a	21.3 ± 3.2 a	4.2 ± 0.6 a	2.9 ± 0.2 b	2.3 ± 0.5 a	2.5 ± 0.4 a	6.9 ± 2.0 a
	0.17	40.0 ± 9.6 b	5.3 ± 0.6 b	8.6 ± 0.8 a	15.6 ± 1.1 a	2.3 ± 0.1 b	2.3 ± 0.5 b	1.7 ± 0.2 a	2.7 ± 0.1 a	5.9 ± 0.7 a
	5.2	576 ± 42 a	7.3 ± 1.2 ab	7.8 ± 1.5 a	17.2 ± 2.4 a	2.0 ± 0.6 b	4.6 ± 0.4 a	2.1 ± 0.3 a	2.3 ± 0.2 a	5.5 ± 1.3 a
Sultan	0	0.33 ± 0.0 b	9.3 ± 0.8 a	19.1 ± 0.6 a	31.4 ± 0.9 ab	2.4 ± 0.3 ab	6.6 ± 0.6 a	3.0 ± 0.2 a	10.0 ± 0.6 a	9.0 ± 0.8 a
	0.17	46.3 ± 25 b	11.6 ± 0.8 a	18.7 ± 1.7 ab	33.4 ± 2.3 a	3.1 ± 0.1 a	8.2 ± 0.7 a	3.1 ± 0.1 a	9.7 ± 1.3 a	9.0 ± 0.5 a
	5.2	745 ± 31 a	8.8 ± 0.5 a	14.6 ± 0.6 b	27.0 ± 1.2 b	2.0 ± 0.2 b	6.5 ± 0.4 a	3.7 ± 0.2 a	7.6 ± 0.3 a	7.0 ± 0.2 a
VI158	0	0.0 ± 0.0 b	11.5 ± 1.0 a	6.2 ± 1.4 a	18.9 ± 2.7 a	6.0 ± 0.4 a	4.2 ± 0.5 a	1.2 ± 0.3 b	5.1 ± 1.3 a	1.1 ± 0.2 a
	0.17	17.7 ± 3.6 b	11.2 ± 0.4 ab	10.5 ± 0.5 a	24.4 ± 1.1 a	5.0 ± 0.5 a	4.9 ± 0.4 a	2.8 ± 0.5 a	7.3 ± 0.9 a	3.1 ± 0.9 a
	5.2	484 ± 117 a	8.5 ± 0.6 b	8.8 ± 1.5 a	19.0 ± 1.1 a	3.0 ± 0.5 b	5.0 ± 0.5 a	1.8 ± 0.3 ab	7.3 ± 1.6 a	1.4 ± 0.1 a
SU003	0	1.0 ± 0.5 b	12.3 ± 1.1 a	8.6 ± 1.0 ab	23.0 ± 2.1 a	2.2 ± 0.1 a	9.8 ± 1.0 a	2.2 ± 0.1 b	4.9 ± 0.5 a	3.4 ± 0.4 b
	0.17	25.0 ± 2.3 b	11.4 ± 0.1 a	7.4 ± 0.8 b	20.6 ± 0.9 a	2.4 ± 0.3 a	8.8 ± 0.4 a	1.9 ± 0.1 b	3.9 ± 0.3 a	3.2 ± 0.5 b
	5.2	604 ± 70 a	6.1 ± 0.9 b	12.8 ± 1.5 a	22.2 ± 1.3 a	1.5 ± 0.3 a	4.5 ± 0.5 b	3.3 ± 0.1 a	4.5 ± 0.4 a	8.1 ± 1.1 a
Brigidier	0	0.67 ± 0.6 b	5.2 ± 1.4 a	6.6 ± 0.3 a	13.2 ± 1.7 a	2.1 ± 0.4 a	2.9 ± 1.0 a	1.5 ± 0.1 a	3.3 ± 0.1 a	3.2 ± 0.2 a
	0.17	51.0 ± 22 b	6.5 ± 1.5 a	2.0 ± 0.5 c	9.5 ± 2.2 a	2.1 ± 0.2 a	4.3 ± 1.3 a	0.9 ± 0.2 b	1.1 ± 0.2 b	0.8 ± 0.2 c
	5.2	193 ±32 a	8.1 ± 0.9 a	3.6 ± 0.2 b	13.2 ± 1.2 a	2.7 ± 0.2 a	5.2 ± 0.7 a	1.5 ± 0.1 a	1.8 ± 0.2 b	1.7 ± 0.1 b
Pirate	0	0.0 ± 0.0 b	7.8 ± 1.2 a	4.8 ± 1.8 a	13.5 ± 2.9 a	2.5 ± 0.2 a	5.0 ± 1.3 a	0.9 ± 0.2 a	3.6 ± 1.4 a	1.2 ± 0.4 a
	0.17	17.7 ± 1.3 b	5.3 ± 0.3 a	6.4 ± 1.8 a	12.8 ± 2.2 a	2.0 ± 0.1 a	3.0 ± 0.4 a	1.1 ± 0.2 a	4.1 ± 1.0 a	2.3 ± 0.9 a
	5.2	374 ± 14 a	6.1 ± 1.5 a	6.7 ± 2.3 a	14.1 ± 3.0 a	1.8 ± 0.4 a	4.0 ± 1.1 a	1.3 ± 0.2 a	3.6 ± 1.1 a	3.0 ± 1.2 a

[x] 4MSOB: 4-methylsulphinylbutyl GS, 3IM: 3-indolymethyl GS, N-3IM: N-methoxy-3-indolylmethyl GS. The total aliphatic and indolyl GS concentrations include the concentration of 3-methylsulphinylpropyl GS and (2R) 2-hydroxy-3-butenyl GS, and 4-methoxy-3-indolylmethyl GS, respectively.

[y] Means with a different letter in each column and genotype are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

Table 5.2. Effect of MeJA and the combination treatment of MeJA with the high Se fertilization treatment on the concentration of GSs (± SE; μM/g DW) in floret tissues of the 5 broccoli genotypes.

Genotype	Treatment [y]	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
					4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Green Magic	Control	9.5 ± 1.0 a [z]	9.5 ± 2.3 b	21.3 ± 3.2 b	4.2 ± 0.6 a	2.9 ± 0.2 a	2.3 ± 0.5 b	2.5 ± 0.4 ab	6.9 ± 2.0 b
	MeJA	10.0 ± 2.2 a	20.1 ± 1.1 a	34.0 ± 2.0 a	5.4 ± 1.4 a	2.2 ± 0.3 a	3.9 ± 0.4 a	2.9 ± 0.3 a	17.1 ± 1.0 a
	MeJA + 5.2 mM Se	6.5 ± 0.3 a	21.3 ± 0.4 a	30.7 ± 0.2 a	2.1 ± 0.2 a	3.3 ± 0.5 a	3.0 ± 0.04 ab	1.7 ± 0.03 b	19.4 ± 0.4 a
Sultan	Control	9.3 ± 0.8 a	19.1 ± 0.6 b	31.4 ± 0.9 b	2.4 ± 0.3 a	6.6 ± 0.6 a	3.0 ± 0.2 c	10.0 ± 0.6 ab	9.0 ± 0.8 b
	MeJA	10.3 ± 0.8 a	29.4 ± 1.6 a	44.6 ± 2.0 a	2.6 ± 0.03 a	7.4 ± 0.8 a	4.9 ± 0.3 ab	10.6 ± 0.7 a	18.4 ± 0.9 a
	MeJA + 0.17 mM Se	9.4 ± 1.4 a	32.2 ± 1.4 a	47.1 ± 2.1 a	4.2 ± 0.8 a	5.0 ± 0.7 a	5.5 ± 0.2 a	7.7 ± 1.1 bc	24.3 ± 1.4 a
	MeJA + 5.2 mM Se	8.4 ± 0.8 a	22.9 ± 1.6 b	35.7 ± 1.3 b	1.7 ± 0.02 a	6.4 ± 0.8 a	4.4 ± 0.4 b	5.9 ± 0.4 c	16.9 ± 2.0 a
VI158	Control	11.5 ± 1.0 a	6.2 ± 1.4 b	18.9 ± 2.7 b	6.0 ± 0.4 a	4.2 ± 0.5 a	1.2 ± 0.3 b	5.1 ± 1.3 b	1.1 ± 0.2 b
	MeJA	10.6 ± 1.0 a	17.1 ± 1.2 a	30.2 ± 0.8 a	3.8 ± 0.5 b	5.7 ± 1.2 a	2.4 ± 0.1 a	13.4 ± 1.2 a	3.6 ± 0.6 a
	MeJA + 0.17 mM Se	9.9 ± 1.2 a	16.6 ± 2.0 a	28.9 ± 3.2 ab	2.6 ± 0.04 bc	6.4 ± 1.1 a	2.4 ± 0.1 a	11.5 ± 2.4 ab	4.9 ± 0.4 a
	MeJA + 5.2 mM Se	5.7 ± 0.9 b	13.1 ± 3.2 ab	20.9 ± 4.2 ab	1.7 ± 0.2 c	3.3 ± 0.6 a	2.1 ± 0.4 ab	10.0 ± 2.6 ab	3.1 ± 0.6 a
SU003	Control	12.3 ± 1.1 a	8.6 ± 1.0 b	23.0 ± 2.1 ab	2.2 ± 0.1 a	9.8 ± 1.0 a	2.2 ± 0.1 ab	4.9 ± 0.5 a	3.4 ± 0.4 b
	MeJA	9.8 ± 0.7 a	21.1 ± 5.2 a	34.4 ± 6.3 a	2.4 ± 0.4 a	7.1 ± 0.9 a	3.5 ± 0.6 a	5.8 ± 1.0 a	14.7 ± 4.3 a
	MeJA + 5.2 mM Se	1.2 ± 0.2 b	5.9 ± 1.2 b	8.1 ± 1.5 b	0.8 ± 0.2 b	0.3 ± 0.1 b	1.0 ± 0.2 b	1.8 ± 0.3 b	4.2 ± 0.9 b
Brigidier	Control	5.2 ± 1.4 ab	6.6 ± 0.3 a	13.2 ± 1.7 a	2.1 ± 0.4 a	2.9 ± 1.0 b	1.5 ± 0.1 a	3.3 ± 0.1 a	3.2 ± 0.2 a
	MeJA	4.3 ± 0.9 b	10.4 ± 2.6 a	16.5 ± 4.0 a	2.2 ± 0.3 a	1.9 ± 0.7 b	1.7 ± 0.5 a	3.6 ± 1.0 a	6.6 ± 1.6 a
	MeJA + 5.2 mM Se	9.0 ± 0.3 a	5.7 ± 0.6 a	16.7 ± 1.1 a	2.3 ± 0.01 a	6.4 ± 0.3 a	2.1 ± 0.4 a	1.7 ± 0.3 a	3.2 ± 0.5 a

([x]: see table 9.1)

[y] Control: treatment with control solution, MeJA: treatment with methyl jasmonic acid, MeJA + 0.17 or 5.2 mM Se: combination treatment of low or high level of Se fertilization with MeJA spray. Samples were harvested 4 days after control or MeJA treatment.

[z] Means within each columm and genotype with different letters are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

Table 5.3. Overall mean GS concentrations (± SE; μM/g DW) across the 5 broccoli genotypes treated with control, low levels of Se, high levels of Se, MeJA, or combination treatment of MeJA with high levels of Se.

Treatment [y]	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
				4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Control	9.6 ± 1.5 a [z]	10.0 ± 4.1 c	21.6 ± 5.4 b	3.4 ± 0.3 a	5.3 ± 1.2 a	2.0 ± 0.6 c	5.2 ± 2.0 b	4.7 ± 1.7 c
0.17 mM Se	9.2 ± 1.6 a	9.4 ± 2.9 c	20.7 ± 4.4 b	3.0 ± 0.6 a	5.7 ± 1.4 a	2.1 ± 0.5 bc	4.9 ± 1.7 b	4.4 ± 1.6 c
5.2 mM Se	7.8 ± 0.9 b	9.5 ± 2.2 c	19.7 ± 2.7 b	2.2 ± 0.4 b	5.2 ± 0.6 a	2.5 ± 0.5 b	4.5 ± 1.4 b	4.7 ± 1.6 c
MeJA	9.0 ± 1.6 ab	19.6 ± 4.0 a	31.9 ± 5.6 a	3.3 ± 0.8 a	4.9 ± 1.4 ab	3.3 ± 0.7 a	7.3 ± 2.2 a	12.1 ± 3.5 a
MeJA + 5.2 mM Se	6.2 ± 1.5 c	13.8 ± 4.1 b	22.4 ± 5.4 b	1.7 ± 0.3 b	4.0 ± 1.2 b	2.5 ± 0.6 b	4.2 ± 2.0 b	9.4 ± 3.9 b

[x] 4MSOB: 4-methylsulphinylbutyl GS, 3IM: 3-indolymethyl GS, N-3IM: N-methoxy-3-indolylmethyl GS. The total aliphatic and indolyl GS concentrations include the concentration of 3-methylsulphinylpropyl GS and (2R) 2-hydroxy-3-butenyl GS, and 4-methoxy-3-indolylmethyl GS, respectively.

[y] Control: treatment with control solution, 0.17 mM Se: low level of Se fertilization, 5.2 mM Se:high level of Se fertilization, MeJA: treatment with methyl jasmonic acid, MeJA + 5.2 mM Se: combination treatment of high level of Se fertilization with MeJA spray. Samples were harvested 4 days after control or MeJA treatment.

[z] Means within each column and with different letters are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

Table 5.4. Percentages of total phenotypic variation described by the regression model in GS concentrations associated with genotype, treatment, and genotype by treatment interaction for 5 broccoli genotypes treated with control, MeJA, high level of selenium, or combination of high level of selenium with MeJA treatment.

[x] 4MSOB: 4-methylsulphinylbutyl GS, 3IM: 3-indolymethyl GS, N-3IM: N-methoxy-3-indolylmethyl GS. The total aliphatic and indolyl GS concentrations include the concentration of 3-methylsulphinylpropyl GS and (2R) 2-hydroxy-3-butenyl GS, and 4-methoxy-3-indolylmethyl GS, respectively.

[y] G × T = genotype × treatment interaction.

[z] R^2 = coefficient of determination.

*, **, ***Significant at P ≦ 0.1, 0.05, or 0.01, respectively.

Source of variation	Total Aliphatic GSs	Total Indolyl GSs	Total GSs	Aliphatic GSs [x]		Aromatic GS [x]	Indolyl GSs [x]	
				4MSOB	3-butenyl	2-phenylethyl	3IM	N-3IM
Genotype	28.5*	81.7***	91.8***	54.0***	49.9***	82.7***	86.0***	78.2***
Treatment (Se)	18.2**	0.3	2.8	20.2***	0.1	7.9**	0.8	0.0
G × T [y]	50.3**	14.2**	3.2	25.2**	48.6***	7.3	9.3*	18.7**
Rep	3.0	3.8	2.1	0.6	1.3	2.1	3.3	3.1
R^2 [z] described by the model	0.65	0.85	0.76	0.80	0.80	0.82	0.85	0.81
Genotype	89.7***	51.3***	63.7***	77.3***	90.7***	61.7***	71.9***	50.0***
Treatment (MeJA)	1.1	43.0***	30.8***	0.2	0.8	32.1***	8.4***	37.5***
G × T	6.1	4.2	3.8	15.6	8.4	6.0	18.7***	3.3*
Rep	1.4	1.4	1.7	7.0	0.1	0.3	1.0	3.3
R^2 described by the model	0.63	0.82	0.80	0.74	0.80	0.80	0.90	0.85
Genotype	6.8	73.4***	73.4***	34.8***	20.3***	71.6***	67.4***	68.0***
Treatment (Se and MeJA)	29.1***	8.6***	0.3	35.1***	6.9***	5.8**	2.4	14.9***
G × T	62.3***	16.5***	25.9***	29.8***	71.2***	20.9**	26.1***	16.2***
Rep	1.8	1.5	0.4	0.3	1.7	1.8	4.1	0.9
R^2 described by the model	0.84	0.88	0.84	0.89	0.88	0.81	0.82	0.94

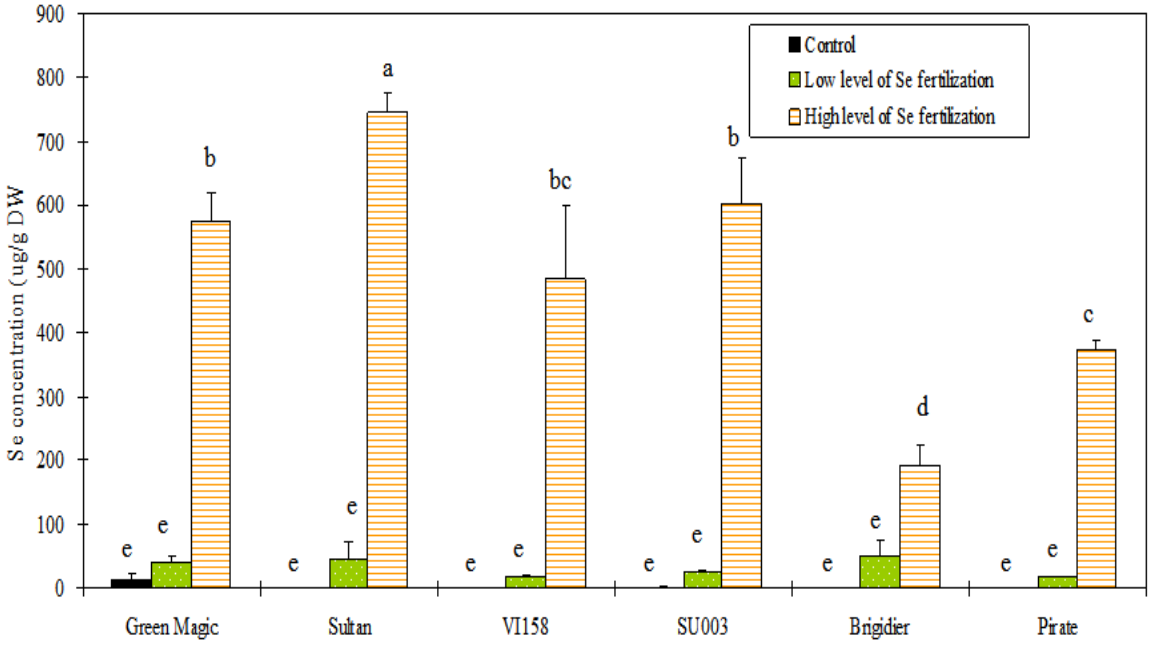


Figure 5.1. Variation in Se concentrations (μg/g DW) among 6 broccoli genotypes in control plants and plants treated with two different levels of Se fertilizer (low or high level of Na_2SeO_4). Means with a different letter are significantly different at P ≦0.05, using Fisher's LSD analysis.

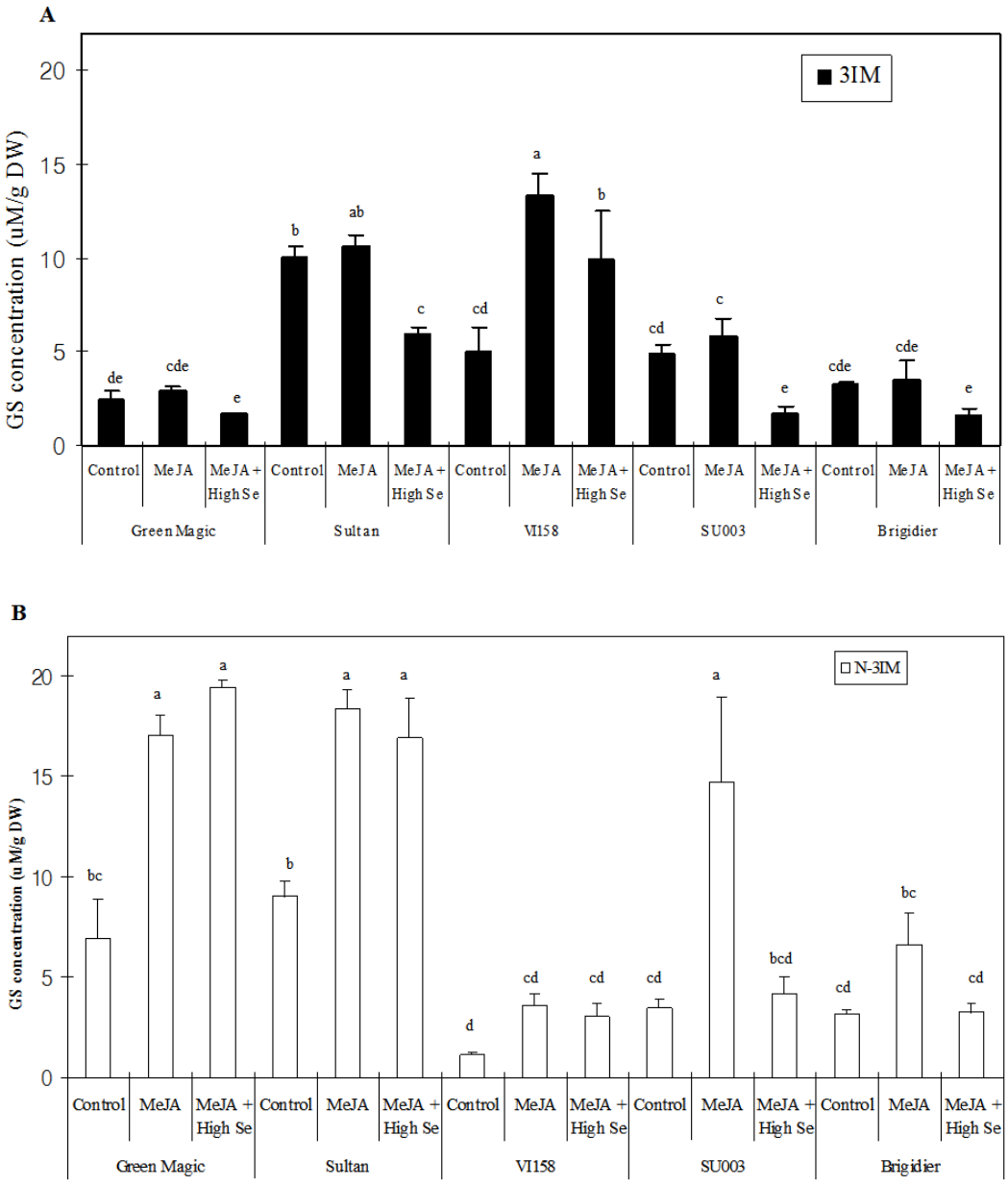


Figure 5.2. Variation in 3-indolymethyl GS (A) and N-methoxy-3-indolylmethyl GS (B) concentrations (μM/g DW) among 5 broccoli genotypes for controls and plants treated with MeJA or combination of MeJA with high levels of Se fertilizer. 3IM is an abbreviation of 3-indolymethyl GS, and N-3IM of N-methoxy-3-indolylmethyl GS. Means with a different letter are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

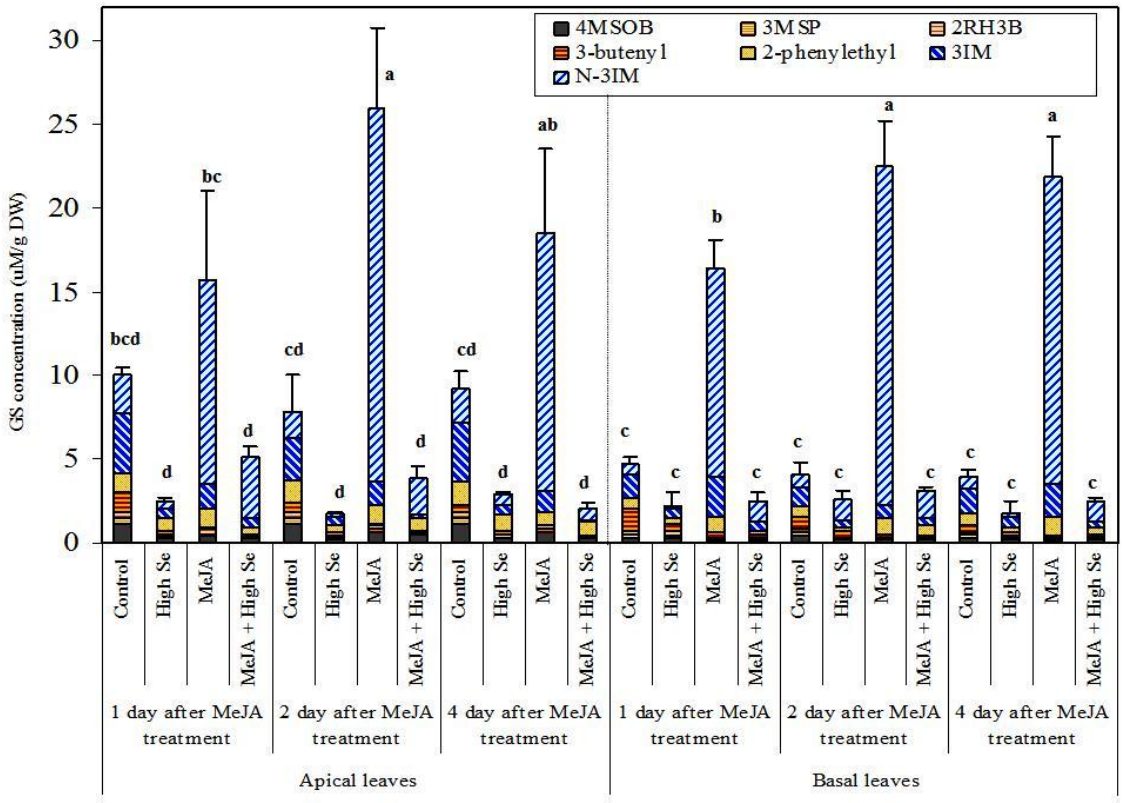


Figure 5.3. Changes in proportions of GS concentrations (μg/g DW) in apical and basal leaves of 'Green Magic' seedlings in controls and plants treated with high levels of Se fertilizer 1, 2 and 4 days after MeJA treatment. 4MSOB is an abbreviation of 4-methylsulphinylbutyl GS, 3MSP of 3-methylsulphinylpropyl GS, 2RH3B of (2R) 2-hydroxy-3-butenyl GS, 3IM of 3-indolymethyl GS, and N-3IM of N-methoxy-3-indolylmethyl GS. Means of total GS concentrations in apical or basal leaves with a different letter are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis. Error bar indicates standard deviation of total GS concentrations.

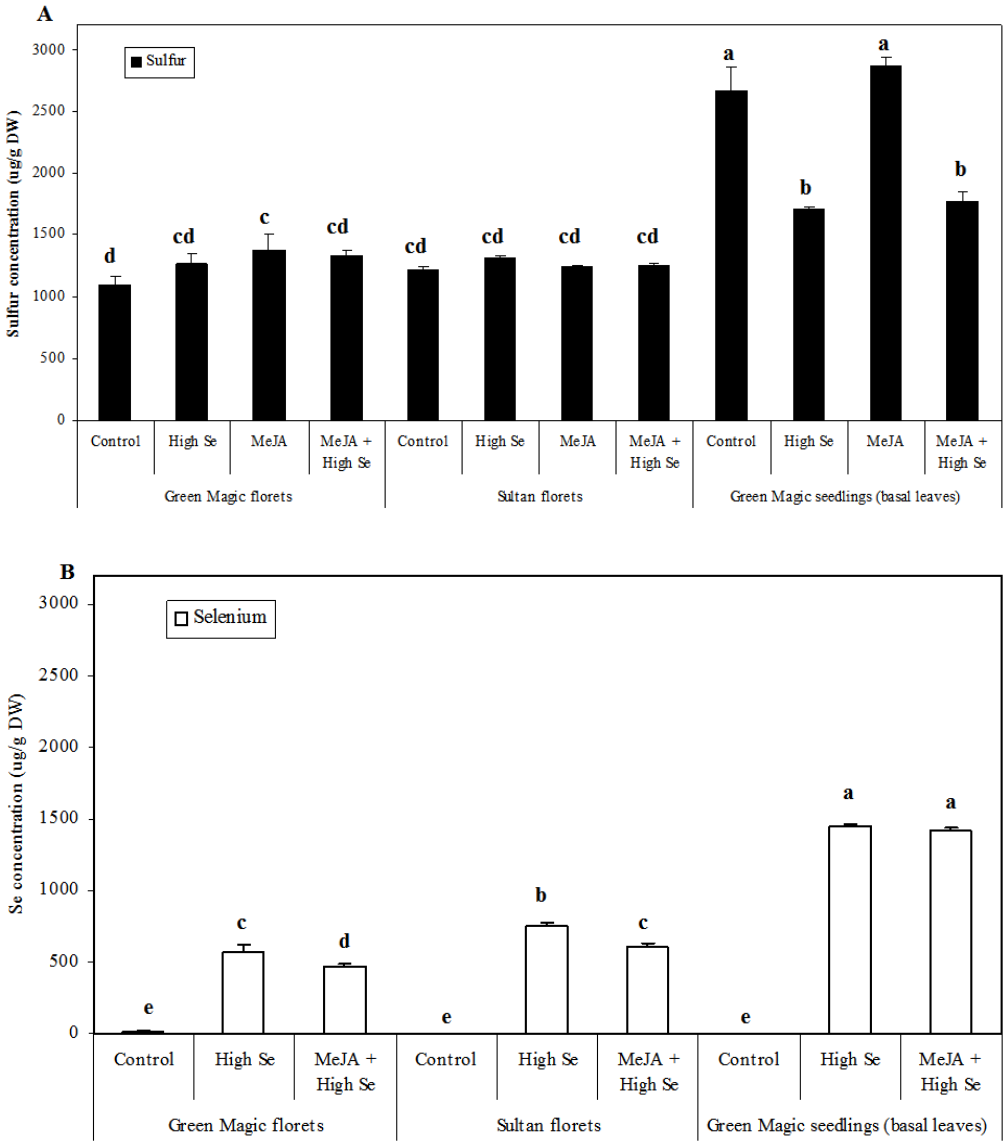


Figure 5.4. Variation in total sulfur (A) and Se (B) concentrations (µg/g DW) in floret tissues of 'Green Magic' and 'Sultan', and in basal leaves of 'Green Magic' seedlings in controls and plants treated with high levels of Se fertilizer, MeJA or the combined treatment. Means with a different letter are significantly different at $P \leqq 0.05$, using Fisher's LSD analysis.

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FUNCTIONAL STUDIES OF LIGNIN BIOSYNTHESIS GENES AND PUTATIVE FLOWERING GENE IN *MISCANTHUS* X *GIGANTEUS*

CHAPTER 6

Literature Review

6.1. Demand for renewable bioenergy production and utilization

Increasing global energy demand, the volatility of the energy prices, and recent geopolitical acts of war and terrorism are main factors aggravating the vulnerability of the current energy supply system that depend on fossil fuels. The growth in global energy demand is projected to rise by more than 50% by 2025, with much of this increase in demand taking place in the rapidly developing countries (Ragauskas et al., 2006). Energy dependency for finite petroleum resources may foster problems including depletion of fossil fuel reserves, energy security concerns and rising energy costs (Asif and Muneer, 2007). Global warming is another critical issue requiring effective management of greenhouse gas emissions. Continuous combustion of fossil fuels and deforestation are considered the major cause of greenhouse effects. The atmospheric carbon dioxide concentration has increased by almost 35% since the beginning of the age of industrialization and is expected to continue increasing (IPCC, 2007). It is crucial to make a transition from our current fossil fuel dependent energy supply chain to renewable and sustainable energy systems. Power generation from bioenergy sources helps to alleviate greenhouse gas emission and reduce use of non-renewable petroleum supplies (Wang et al., 1999). Currently, 80% of total global energy supplies depend on fossil fuels such as coal, gas and petroleum-based oils. Renewable energy sources represent only 13% of total primary energy supplies with biomass dominating at 10 % in renewable sector (IEA, 2007).

6.1.1. Bioenergy

Bioenergy is renewable energy made from biological sources that can be used for heat, electricity and fuel, and their co-products. Bioethanol, biodiesel and biogas are the three major

bioenergy products. Current liquid biofuels derived from plant sugars, starch, and oil are referred to as first-generation biofuels (FAO, 2008). Currently, over 10 billion gallons of bioethanol is produced globally per year from corn starch and sugar (sugarcane and sugar beets) through conventional technologies involving hydrolysis of starch and fermentation of sugar (Rass-Hansen et al., 2007; Goldemberg, 2007). Although the production of ethanol from starch and sugar represents the most convenient and technically advanced option for bioenergy, it results in competition between energy and food supplies, which is not sustainable in the long term (Farrell et al., 2006; De Oliveira et al., 2005).

Projected second generation biofuels will be produced from lignocellulosic biomass from agricultural by-products such as wheat and corn stalks, wood chips, and from energy-dedicated biomass crops through deconstruction of cell wall polymers into fermentable sugar. Lignocellulosic material is more resistant to hydrolysis and fermentation than plant starch and sugar. The resistance of lignocellulose to deconstruction makes the conversion cost more expensive, although the cost of the lignocellulosic biomass itself is lower than for current first-generation feedstocks. Lignocellulosic biofuels are still awaiting development of advanced technology enhancing conversion efficiency of biomass into liquid fuels (FAO, 2008). Third generation biofuels are also slated to be made from non-food crop sources using low input/high output production organisms such as algae (Demirbas, 2009).

6.1.2. Bioenergy feedstocks

The U.S. Department of Energy has set a goal to replace 30% of the liquid petroleum used as fuels for transportation with biofuels and to replace 25% of industrial petroleum-based organic chemicals with those derived from biomass by 2025 (Ragauskas et al., 2006). Substitution of 30% liquid petroleum with ethanol from biomass will require 1 billion tons of

plant biomass annually (Perlack et al., 2005). In this 'billion tons scale' vision, identification and growing of ideal bioenergy crops having high biomass productivity with minimum agricultural input are crucial to fulfill sustainability of the bioenergy supply chain without disruption of food production (Heaton et al., 2008). Ideal bioenergy crops should fulfill the following requirements; highly favorable net energy balance, maximum efficiency in conversion of solar energy into biomass, and high water and nitrogen use efficiency, while providing environmental and ecological benefits (Heaton et al., 2004; Yuan et al., 2008). C_4 perennial rhizomatous grasses suitable for production in temperate regions approach these specified ideals compared to traditional cereal crops (maize and sorghum), sugar-producing crops (sugarcane and sugar beet), and woody perennial species (populus) (Long et al., 1994). Starch-rich cereal crops have a number of disadvantages as energy crops, due to competition with our food supply and requirements for large agricultural input in cultivation and re-planting each year (Hulsbergen et al., 2001). Woody perennials require several to many years between planting and harvest, with the coverage under production not easily converted back to arable use (Heaton et al., 2004). There are two groups of C_4 rhizomatous perennial grass species that have received the most attention as potential biomass crops in Europe and North America; switchgrass (*Panicum virgatum*) and *Miscanthus* (*Miscanthus* spp.).

6.2. *Miscanthus*

Miscanthus is classified taxonomically with maize, sorghum and sugarcane in the grass tribe Andropogoneae, subtribe Saccharinae (Hodkinson et al., 2002). Most of the relevant species of the genus *Miscanthus* are endemic to eastern or south-eastern Asia from temperate and tropical regions (Greef and Deuter, 1993). Among the various *Miscanthus* species, attention has focused on *Miscanthus sinensis*, *M. sacchariflorus*, and *M.* x *giganteus* as a potential bioenergy

crop species (Jones and Walsh, 2001). In eastern Asia accessions of *M. sinensis* are typically diploid (Lafferty and Lelly, 1994). However, there are also natural and artificially induced examples of polyploidy in *M. sinensis* such as the triploid variety known as 'Goliath' (Matumura et al., 1985). In the wild *M. sacchariflorus* is typically either diploid or tetraploid, with accessions ranging from diploid to hexaploid (Hodkinson et al., 1997; Hodkinson et al., 2002). *M.* x *giganteus* is a sterile allotriploid (3N=57) hybrid putatively generated from the natural cross between a diploid *M. sinensis* (2N=38) and a tetraploid *M. sacchariflorus* (4N=76) (Greef et al., 1997).

6.2.1. *Miscanthus* x *giganteus* as a potential energy crop

The C_4 perennial rhizomatous grass species, *M.* x *giganteus* is a potential dedicated bioenergy crop due to its high biomass yield (Heaton et al., 2004), efficiency in converting radiation to biomass (Beale et al., 1996), and high nitrogen (N) and water use efficiency (Beale and Long, 1997; Beale et al., 1999). The capability of *M.* x *giganteus* to reallocate minerals and other nutrients to underground rhizomes as the plant undergoes senescence in the fall minimizes need for application of fertilizers. The rhizomes produce new shoots each spring. Rhizome propagules develop into mature stands in 3-4 years providing maximum yields which can be maintained for over 20 years of production (Lewandowski, 2000). Plantings in Europe over 25 years old have continued annually to provide high biomass yields with minimal input costs.

M. x *giganteus* also has a longer growing season in cool climates compared with maize (*Zea mays*), because of its capability of maintaining photosynthetically active leaves at a temperature 6°C below the minimum for maize (Wang et al., 2008). These and other characteristics make *M.* x *giganteus* a viable bioenergy crop which can be used to generate heat, power and fuel, while reducing carbon dioxide emissions (Heaton et al., 2004). Recent studies in

the US Midwest have shown that *M.* x *giganteus* yielded harvestable biomass of up to 61 t ha^{-1} and averaged 30 t ha^{-1} across three locations, much greater than that observed in a regionally adapted switchgrass cultivar (10 t ha^{-1}) (Heaton et al. 2008).

M. x *giganteus* has limitations for conventional breeding due to the difficulty in generating viable seed from sexual hybridization between the *Miscanthus* parental species and sterility of triploid accessions. Difficulties in sexual hybridization have restricted the development of useful genetic diversity for breeding improvement. A single clonally propagated genotype accounts for nearly all commercial production. As an unimproved crop, obtaining genetic variation is required to select genotypes with increased yield and quality, and to provide diversity that will be needed as a source of resistance to pests and diseases that may arise in the future (Clifton-Brown *et al*. 2001; 2008).

6.3. Micropropagation of *M.* x *giganteus*

As a naturally occurring sterile hybrid, *M.* x *giganteus* does not form seeds and must be propagated vegetatively by mechanically divided rhizomes or by micropropagation of plantlets. This requirement makes the establishment of sterile triploid *M.* x *giganteus* plantings costly and labor intensive (Lewandowski et al, 2000). The estimated cost for mechanization of rhizome establishment was 350 Euro ha^{-1} ($128 $acre^{-1}$) with expectation for costs to be reduced to 200 Euro ha^{-1} or about US $74 $acre^{-1}$ in the future. Micropropagated plants, produced by *in vitro* culture, require much higher establishment costs of about 3000-6000 Euro (US$2730-5460) per hectare for typical densities of one or two plants per square meter (Lewandowski et al., 2000). The cost of plant propagules and the need to propagate large numbers of plants are key factors constraining widespread planting of *M.* x *giganteus* (Atkinson, 2009). Micropropagation could provide for the rapid multiplication of initial germplasm stocks selected in breeding programs.

These materials would then undergo rhizome propagation to fullfill the requirement of large-scale establishment in the field. Micropropagation is also a viable approach for non-seasonal production, production of disease-free plants, germplasm conservation and to facilitate international exchange (Govil and Gupta, 1997). Increasing demand of *M.* x *giganteus* cultivation in various regions and countries requires improvement of current tissue culture micropropagation techniques to reduce propagule cost and increase availability.

In vitro propagation of *M.* x *giganteus* has been conducted by shoot regeneration from axillary nodes and apical meristems followed by *in vitro* tillering (Nielsen et al., 1993; Nielsen et al., 1995; Lewandowski, 1997), and by plantlet regeneration through somatic embryogenesis of callus induced from shoot apices, leaf sections and immature inflorescence tissues (Lewandowski, 1997; Holme and Petersen, 1996). Previous investigations have focused on optimization of tissue culture conditions to improve the induction rate of the embryogenic callus, described as a compact opaque white callus, by addition of proline and different carbon sources, and the use of liquid suspension culture for *M.* x *giganteus* (Holme et al., 1997; Petersen et al., 1999). Combination of auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (benzyladenine) in callus induction medium increased the frequency of both embryogenic and shoot-forming callus from leaf sections and shoot apices compared with the medium containing auxin alone (Petersen, 1997). Relatively high regeneration rates of *M.* x *giganteus* callus was also achieved by the use of 2,4-D and BA when callus is induced from immature inflorescence tissues (Lewandowski, 1997).

6.4. Plant cell walls

Cellulose, hemicellulose and lignin are the major components of plant cell walls. The main structural component of plant cell wall is cellulose composed of a long chain of glucose

molecules linked to one another primarily by β (1→4) glycosidic bonds (van Wyk, 2001). Hemicellulose is the second most abundant constituent of plant cell wall. Hemicelluloses, consisting of polymers of xylose, arabinose, galactose, mannose and glucose (5- and 6-carbon monosaccharide units) link bundles of cellulose fibres to form microfibrils, and also cross-link with lignin, creating a complex network of bonds that provide structural strength to the plant (van Wyk, 2001).

6.4.1. Lignin

Lignin is a necessary component for the structural integrity of plants, conduction of water, and for protection from pests and pathogens (Campbell and Sederoff, 1996; Kenrick and Crane, 1997). Lignins, complex aromatic heteropolymers, are derived from three hydroxycinnamyl alcohol monomers, *p*-coumaryl, coniferyl, and sinapyl alcohols. Distinguished by their degree of methoxylation, these monolignols produce *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) constitutents, respectively. These three lignin monomers synthesized via the concerted action of the shikimic acid and phenylpropanoid pathways are transported to the cell wall, and undergo polymerization through an oxidative coupling mechanism.

Lignin forms covalent bonds with cellulose and hemicellulose polysaccharides in cell walls, and this structure inhibits enzyme access by cellulases during the biomass degradation process prior to fermentation. Lignin also inhibits microbial activity needed for the fermentation of cell-wall hydrolysates to ethanol. The low efficiency of conversion from biomass to fermentable sugar and the coproduction of toxic compounds in the presence of high lignin concentration increase the expense of pretreatment and enzyme costs. The amount and composition of lignins vary among cell wall types and layers, and the specific composition is directly related to physiological function such as vascular integrity of a particular cell type within

the vascular apparatus (Reviewed in Boerjan et al., 2003). In general, G and S units are the main components of dicotyledonous angiosperm (hardwood) lignins, whereas gymnosperm (softwood) lignins consist mostly of G units with low levels of H units. Grasses contain G and S units at comparable levels with more H units than dicots (Baucher et al., 1998).

6.4.2. Lignin biosynthetic pathway and gene expression

The monolignol biosynthetic pathway has been characterized by *in vitro* experiments testing the conversion of intermediate compounds by enzymatic reactions at each metabolic stage (Anterola and Lewis, 2002). Transgenic plants or mutants with modified expression of monoligniol biosynthesis genes have been used to reveal the function of the genes encoding each enzyme of the lignin metabolic networks (Figure 6.1). The entry point enzyme to the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL), was thought to be an important first target to reduce carbon flow into the monolignol biosynthetic pathway. *PAL* downregulated tobacco plants showed a number of abnormalities such as stunted growth, deformed flowers, and leaf epinasty with highly decreased overall lignin content (Bate et al., 1994). Impaired vascular integrity of *PAL* downregulated plants is presumable due to the reduced lignin level. However, it is still not clear if other developmental abnormalities such as distorted growth patterns are due to downstream reduction in lignin content. The many functions of PAL involved in different metabolic processes suggest that the modification of this enzyme may cause unpredictable physiological changes not directly related to downstream reduction of lignin.

Cinnamate 4-*hydroxylase* (C4H), p-*coumarate* 3-*hydroxylase* (C3H), and *ferulate* 5-*hydroxylase* (F5H) are cytochrome P450-dependent monooxygenases, mainly involved with conversion of cinnamate into 4-hydroxy-cinnamate, introduction of a hydroxyl group into the *meta*-position of phenylpropanoids, and hydroxylation at C-5 on the aromatic ring providing the

substitution pattern necessary for S unit formation, respectively (Figure 6.1). The function of these *P450* genes was investigated using transgenic plants transformed with antisense genes or knockout mutant lines in *Arabidopsis* (Franke et al., 2002), tobacco (Blount et al., 2000) and *Populus* (Lu et al., 2006). Interestingly, down-regulation of the *C4H* gene by antisense constructs induced substantial reductions in lignin content, but no visible abnormalities in the growth and development of transgenic tobacco (Sewalt et al., 1997; Blount et al., 2000). The lignified regions in transgenic plants were similar to wild-type plant, although staining intensity was diminished in plants transformed with *C4H* antisense constructs. These results suggest that *C4H* is a target for the progressive and quantitative reduction in lignin content without serious compositional change. *C4H* is encoded at a single locus in *Arabidopsis* and pea (Bell-Lelong et al, 1997; Frank et al., 1996), whereas it has a small gene family in alfalfa (Fahrendorf and Dixon, 1993), mung bean (Mizutani et al., 1993), and *Catharanthus roseus* (Hotze et al., 1995). Expression pattern of *C4H* mRNA was analyzed in *Arabidopsis* by RNA blot hybridization and *C4Hpro-GUS* fusion assay (Bell-Lelong et al, 1997). The highest levels were found in stem and root tissue, and the accumulation of *C4H* mRNA was substantially enhanced in light-grown seedlings and wounded tissue. GUS staining was strongly localized to the vein of the mature leaves and restricted to the xylem and parenchyma in stem cross-sections. Consistent with *C4H* mRNA accumulation, *C4H*-driven GUS was also induced by wounding. *C4H* activity was also induced by a number of treatments such as light, wounding and the application of exogeneous p-coumaric acid (Bolwell and Dixon, 1986; Lamb and Rubery, 1976). There is no *Arabidopsis* mutant defective in *C4H* activity. It is supposed that loss-of-function of *C4H* would be lethal due to the impact of the mutation on vascular development. *C4H* promoter of *Populus* was also characterized by GUS fusion assay (Zhao et al., 2005). A putative *C4H* promoter fragment was

isolated from genomic DNA of *Populus tomentosa* by PCR, and *PC4Hpro-GUS* construct was introduced to tobacco plants. Histochemical GUS analysis showed that the expression of *C4H-GUS* fusion product is mainly localized in the lignified tissues and its activity gradually increased from the first to the ninth internode in the stem of tobacco. Over-expression and down-regulation experiments for the *C4H* gene were conducted in tobacco transformed with sense or antisense constructs containing the 35S promoter and alfalfa *C4H* cDNA (Sewalt et al., 1997). The reduction of *C4H* activity by antisense expression resulted in reduced levels of Klason lignin, accompanied by a decreased syringyl/guaiacyl monomer ratio. Treatment with safranin-O and astra-blue which stains lignin and cellulose fibers, respectively, confirmed that with the downregulation of *C4H*, changes in color of distinct rows in the vascular tissue was observed with effects not evenly distributed over different cell types. *C4H* genes that exist as a multigene family in *Populus* were isolated and identified to understand the roles of individual *C4H* members (Lu et al., 2006). Two *C4H* cDNA (*PtreC4H*) isolated from *Populus tremuloides* and three *C4H* genes (*PtriC4H*) identified in the *P. trichocarpa* showed different expression patterns in various tissues, and these differential expresson patterns of *C4H* genes were associated with cis-acting regulatory elements such as L, P and H boxes.

C3H, one of the P450 family (CYP98A3), is also a key enzyme participating in the formation of G and S monolignol units. The *C3H* gene is present in the *Arabiodpsis* genome as a single copy, and the function of this gene was identified and characterized by knock-out mutants and promoter assays (Franke et al., 2002; Nair et al., 2002; Schoch et al., 2006). The expression pattern of the *C3H* gene was similar to that of the *C4H* gene (Schoch et al., 2001). Highest expression was detected both in stems and roots associated with lignifying tissues. *C3H* specific expression was also analyzed during *Arabidopsis* development by promoter-GUS fusions. The

C3H promoter showed more restricted tissue-specific expression compared to *C4H* (Nair et al., 2002). *C3H* was not expressed in the seeds and only expressed in the vascular tissue of petals, sepals, anthers, and stigmas, whereas *C4H* showed an overall expression in flower tissues and a very intense expression in seed. A mutation of *C3H* gene in *Arabidopsis* displayed a dwarfed phenotype with ~20% lower lignin content than the wild type by the thioglycolic acid extraction method and ~40% of control as measured as Klason lignin (Franke et al., 2002). This mutant possessed only H units, whereas wild type plants have only trace levels. This indicates that the pathways to both coniferyl and sinapyl alcohol were blocked, and H units were made to compensate for missing G and S units in these *C3H* mutants. These results demonstrate that the suppression of *C3H* has the effect of restricting carbon flow into the monolignol and lignin pathway, and has a significant effect on lignin content and assembly. Since alfalfa *C4H* and *C3H* antisense lines deriven by the *PAL2* promoter showed improved digestibility with reduced lignin content without serious phenotypic changes, the early pathway genes such as *C4H* and *C3H* are considered as a viable target to achieve improvement of biomass quality by genetic modification. The application of tissue specific promoter such as *PAL2* is also considered as an effective approach to avoid deleterious effects such as a dwarfed phenotype caused by the constitutive suppression of lignin biosynthesis genes.

Down-regulation of *4-coumarate: coenzyme A ligase* (*4CL*) led to a reduction of lignin content in tobacco, *Arabidopsis,* and aspen, but their phenotypes displayed significant species-specific differences (Hu et al., 1999; Kajita et al., 1997; Lee et al., 1997). *4CL* down-regulation in aspen showed increased growth with no visible phenotypic change, and dwarfed growth was detected in *Arabidopsis* and tobacco. These results indicate that different species may have differential intrinsic capabilities in tolerating large variations in lignin content and composition.

Reduction in lignin in cell walls of transgenic plants can be compensated for by an increase in other compounds that can be incorporated as an integral component of polymeric lignin (Ralph et al., 1998). Since all *4CL* downregulated plants were generated by antisense constructs, differently suppressed levels of the *4CL* gene might be another reason of species-specific differences in transgenic phenotypes in these species. Hence, greater suppression of the *4CL* gene using RNAi silencing could be used to characterize gene function.

Transgenic down regulation of downstream enzymes in the monolignol pathway (CAD, COMT, and CCR) displayed relatively minor reductions in lignin content, whereas the downregulation of upstream enzymes in this pathway resulted in a more dramatic decrease in lignin contents (Chen et al., 2006). Simultaneous modification of multiple downstream enzymes showed additive reductions in lignin content (Pinçon et al., 2001). The suppression of multiple downstream genes has been simultaneously achieved either by hybridization of single gene transformants [*COMT* and *CCR* (Pinçon et al., 2001), *CAD* and *cinnamoyl-CoA reductase* (*CCR*) (Chabannes et al., 2001)] or by double transformation events [*COMT* and *caffeoyl CoA O-methyltransferase* (*CCoAOMT*) (Guo et al., 2001)].

The *brown midrib* mutations (*bm1, bm2, bm3,* and *bm4*) in maize are associated with significant alterations in lignin composition and content in stover (Petersen et al., 2005). The *bm1* mutation reduces cinnamyl alcohol dehydrogenase (CAD) activity (Haplin et al., 1998) while *bm3* results in reduced caffeic acid O-methyltransferase (COMT) activity (Vignols et al., 1995). Reduction in lignin content of maize has recently been accomplished by down-regulation of *COMT* using antisense constructs, with transgenic plants showing very similar characteristics to the maize *bm3* mutant phenotype (Piquemal et al., 2002). Down-regulation of *COMT* to low activity levels also reduces lignin content in alfalfa, maize, and poplar by 30%, 30%, and 17%,

respectively (Guo et al., 2001; Chabbert et al., 1994; Jouanin et al., 2000). The phenotypes of maize *brown midrib* mutants provide useful information to estimate the potential impact of lignin reduction in grass species sharing similar stalk structures. Although maize *bm3* mutants showed relatively normal phenotypes, this mutation had an unfavorable effect on maize stalk rigidity. Stem stalk strength of *bm3* mutants was reduced (17-26% decrease of crushing strength and 8-14% decrease of stalk-section weight) resulting in maize plants prone to stalk collapse and lodging (Zuber et al., 1977). Sorghum *brown midrib* (*bmr*) mutants generated via chemical mutagenesis of seeds also show lower lignin content with reduced vigor and grain yield (Porter et al., 1978). Selection of the most suitable mutant having the lowest lignin content with highest fiber digestibility without a reduction in overall biomass yield was possible among the various *bmr* mutant lines (Oliver et al., 2005). These results emphasize the importance of mutant pools having variation in lignin content and developmental phenotypes in order to select commercially valuable genotypes as biofuel crop resources.

6.5. Flowering of *Miscanthus*

Although *M.* x *giganteus* is a sterile triploid (Greef and Deuter, 1993), it typically flowers in autumn sending up multiple showy inflorescences from each clone. Flower initiation of *M. sinensis* is day length neutral and appears to be associated with accumulated heat units (Jorgensen & Muhs, 2001). Flowering initiation time in *M. sacchariflorus* is more complex. Some genotypes appear day length-sensitive, whereas others were similar to *M. sinensis* (Clifton-Brown et al., 2008). The completion of flowering in *Miscanthus* species has been associated with the initiation of plant senescence (Mutoh et al., 1968) although low temperatures may also play a role. As with most grasses, flowering interferes with vegetative growth and consequently reduces biomass yield potential due to its termination of vegetative growth and energy loss. This

information suggests that manipulation of genes that regulate the process of flowering induction in *Miscanthus* may be candidates for genetic engineering to develop transgenic genotypes with altered flowering response and enhanced biomass productivity. There is no available information about signaling pathways and key genes associated with *Miscanthus* flowering. Artificial regulation of *Miscanthus* flowering time may also help to make new crosses between diverse *Miscanthus* species for trait improvement through the synchronization of flowering.

6.5.1. Flowering time pathway in monocotyledonous plant

Molecular basis for transition from vegetative to reproductive phase was well studied in the model plant species *Arabidopsis thaliana* (Boss et al., 2004; Henderson and Dean, 2004). Flowering time pathways can be classified as photoperiodic, autonomous, vernalization-induced, and gibberellin-sensitive. In response to environmental or endogenous signals, flowering time genes control transition from vegetative to reproductive growth through each flowering time pathway. All signaling generated from the various flowering time pathways are integrated and lead to the activation of meristem identity genes that specify floral identity through the ABC model (Jack, 2004).

The completion of the genome sequencing project in rice stimulated comparative research on flowering time pathways and related genes between rice, a short-day plant with no vernalization requirement, and *Arabidopsis*, a long-day plant which is vernalization-induced. This research between rice and *Arabidopsis* displayed the distinct differences in response to day-length for flowering (Kojima et al., 2002). *Arabidopsis CONSTANS* (*CO*) gene promoted flowering under long-day conditions by activation of *FLOWERING LOCUS T* (*FT*) mRNA expression, whereas *Heading dates1* gene (*Hd1*), the rice otholog of *CO*, inhibited flowering by repression of *Hd3a*, an otholog of *FT* in *Arabidopsis* under the same photoperiodic conditions.

The rice *Hd1* gene promoted flowering by activation of *Hd3a* under short-day conditions (Izawa et al., 2002). Similar functions of othologous flowering time genes were also observed in maize. A putative maize *CO* otholog (*conz1*) gene was differentially expressed in a circadian pattern (Miller et al., 2008). Among a large maize *FT*-related gene family called *ZCN* (*Zea CENTRORADIALIS*), *ZCN8* exhibited leaf-specific expression patterns and physically interacted with a shoot meristem-localized FD-like protein suggesting a florigenic function of *ZCN8* as a gene orthologous to *FT* in maize (Danilevskaya et al., 2008). These results indicate that, although major genes associated with photoperiodic flowering pathway are conserved among distantly related species, flowering of each species requires distinctly different environmental conditions (Colasanti and Coneva, 2009). Further, the molecular dissection of flowering pathways in *Arabidopsis* and rice provides a useful conceptual framework which allows the identification of othologous flowering time genes whose biological functions can serve as models in other plant species (Cockram et al., 2010).

Some unique genes that appear to be absent in *Arabidopsis* were identified in rice. *Early heading date1* (*Ehd1*), which encodes a B-type response regulator, induce *Hd3a* expression under short day conditions, even in the absence of *Hd1* (Doi et al., 2004). *INDETERMINATE1* (*ID1*) gene, which encodes a zinc-finger protein, also showed no clear *ID1* ortholog in *Arabidopsis* (Colasanti et al., 2006). The *indeterminante 1* (*id1*) mutant in maize showed dramatically delay flowering with extended vegetative growth periods (Colasanti et al., 1998). The maize *ID1* gene was mainly expressed in immature leaves but not at the shoot apex. This result suggests that ID1 regulates the production and transmission of a leaf-derived florigenic signal. The invariability of *ID1* transcript and ID1 protein levels in response to diurnal light changes also suggests that *ID1* gene acts in the autonomous flowering pathway (Wong and

Colasanti, 2007). Orthologous function of *ID1* was also observed in rice (Wu et al., 2008; Matsubara et al., 2008; Park et al., 2008). Similar to maize, rice *ID1* acted as a key regulator of the flowering transition. Rice *ID1* was highly expressed in developing leaves and this expression was unperturbed under diurnal day/night cycles. Rice *ID1* also acted upstream of *FT* ortholog *Hd3a* and the unique *Ehd1* gene. In rice, an *ID1* knockout mutant (*rid1*), referred to as a never-flowering phenotype, completely repressed *Hd3a* and *Ehd1* expression, with partial reduction in the expression of *Hd1* (*CO* ortholog) (Wu et al., 2008). The never-flowering phenotype of this mutant was maintained under inductive short day conditions suggesting that rice *ID1* acts as the master regulator of flowering for both the photoperiod and autonomous pathway.

Mutations associated with flower induction in maize provide a good source of candidate genes for *Miscanthus* since these species are taxonomically related and have very similar flowering morphology. In fact, Vollbrecht et al. (2005) reported that a gene that regulates floral branching in maize (*ramosa1*) shared sequence homology and comparable gene expression patterns with an otholog in *Miscanthus sinensis*. This group was successful at recovering the *Miscanthus* otholog of this maize gene using reverse transcription-PCR (RT-PCR). The *indeterminant 1* (*id1*) mutant in maize is an ideal candidate since a loss-of-function mutation at this locus was found to dramatically delay flowering and extend the vegetative growth period (Colasanti et al., 1998). Homozygous *id1/id1* mutants were much taller plants with more leaves, greater vegetative biomass and no or greatly delayed floral development. Isolation and expression pattern analysis of orthologous *ID1* gene as well as other flowering time genes in *Miscanthus* will provide useful information to understand the role of *ID1* and related flowering signaling pathways in *Miscanthus*.

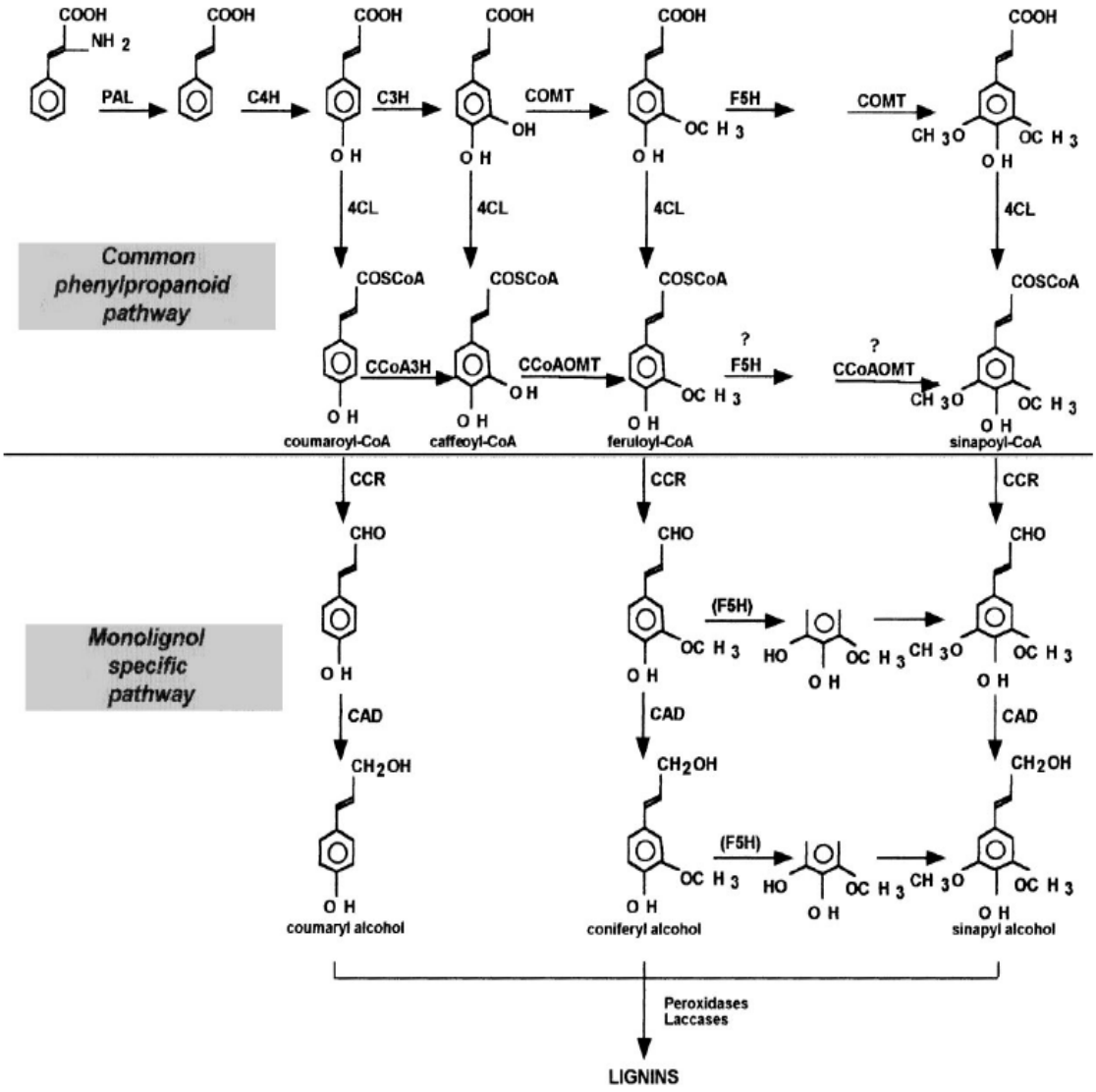


Figure 6.1. Phenylpropanoid and monolignol biosynthetic pathways. Horizontal reactions are ring modification; vertical reactions are side-chain modifications. [Figure source: Boudet, 2000]

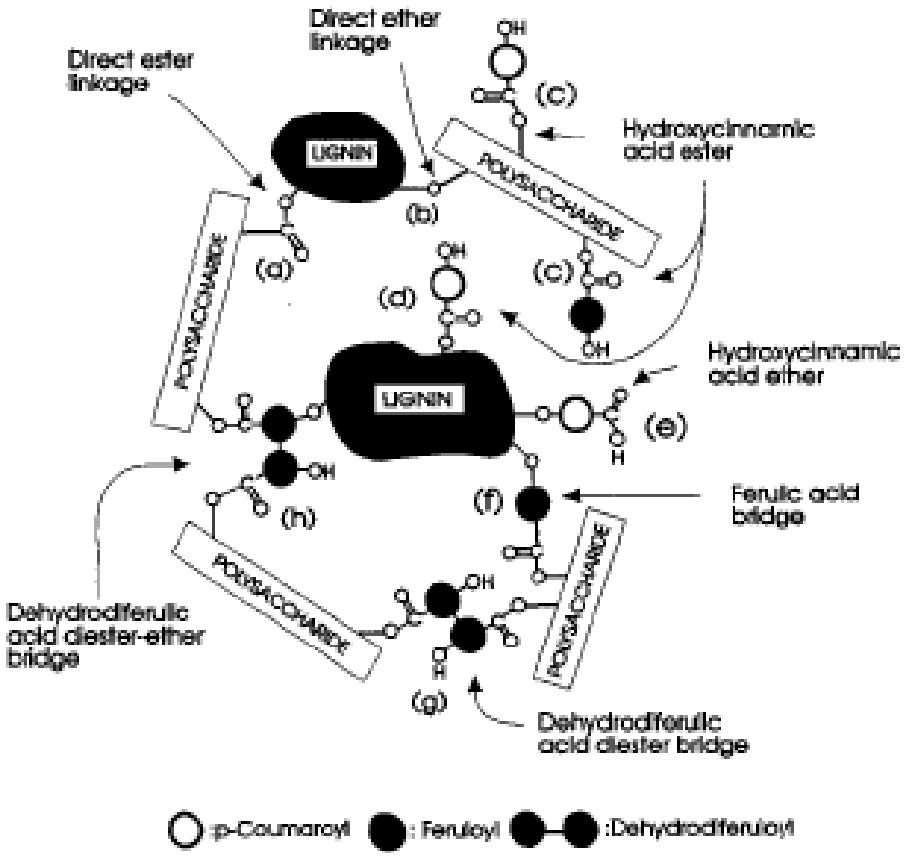


Figure 6.2. Cross-links between lignin and polysaccharides. [Figure source: Liyama et al., 1994]

CHAPTER 7

Miscanthus x *giganteus* plant regeneration: effect of callus types, ages and culture methods on regeneration competence

This Chapter is adapted from: Kim, H.S., Zhang, G., Juvik, J.A., and Widholm, J.M. 2010. *Miscanthus* x *giganteus* plant regeneration: effect of callus types, ages and culture methods on regeneration competence. GCB Bioenergy 2:192-200.

7.1. Abstract

The perennial rhizomatous grass, *Miscanthus* x *giganteus* is an ideal biomass crop due to its rapid vegetative growth and high biomass yield potential. As a naturally occurring sterile hybrid, *M.* x *giganteus* must be propagated vegetatively by mechanically divided rhizomes or from micropropagated plantlets. Plant regeneration through somatic embryogenesis is a viable approach to achieve large-scale production of plantlets in tissue culture. Effect of the callus types, ages and culture methods on the regeneration competence was studied to improve regeneration efficiency and shorten the period of tissue culture in *M.* x *giganteus*. Shoot-forming calli having a yellow or white compact callus with light-green shoot-like structures showed best regeneration frequency. Percentage of shoot-forming callus induction from immature inflorescence explants was 41 % on callus induction medium containing 13.6 μM 2,4-D and 0.44 μM BA. The use of a regeneration medium containing 1.3 μM NAA and 22 μM BA was effective to shorten the incubation period required for plantlet regeneration from shoot-forming calli and 69 % of total regenerated plantlets were obtained within one month of incubation on regeneration medium. Embryogenic-like callus morphotype could maintain regeneration competency for up to one year as suspension cultures. Field grown regenerated plants showed normal phenotypic development with DNA content and plant heights comparable to rhizome propagated plants. Winter survival rates of the regenerated plants planted in 2006 and 2007 were 78 % and 56 %, respectively.

7.2. Introduction

The C_4 perennial rhizomatous grass species, *Miscanthus* x *giganteus* is a sterile allotriploid (3N=57) hybrid putatively generated from the natural cross between a diploid *M. sinensis* (2N=38) and the tetraploid *M. sacchariflorus* (4N=76) (Greef et al., 1997). *M.* x *giganteus* is a potential dedicated bioenergy crop due to its high biomass yield (Heaton et al., 2004), conversion of solar radiation to biomass (Beale et al., 1996), and high nitrogen (N) and water used efficiency (Beale and Long, 1997; Beale et al., 1999). The capability of *M.* x *giganteus* to reallocate minerals and other nutrients to underground rhizomes in the fall minimizes need for fertilizer. The rhizomes produce new shoots annually and generate mature stands in around four years providing maximum yields which can be maintained for over fifteen years of production (Lewandowski, 2000). *M.* x *giganteus* also has a longer growing season in cool climates compared with maize (*Zea mays*), because of its superior capability to maintain photosynthetically active leaves at a temperature 6℃ below the minimum for maize (Wang et al., 2008). These and other characteristics make *M.* x *giganteus* a viable bioenergy crop which can be used to generate heat, power and fuel, while reducing carbon dioxide emissions (Heaton et al., 2004).

As a naturally occurring sterile hybrid, *M.* x *giganteus* does not form seeds and must be propagated vegetatively by mechanically divided rhizomes or micropropagated plantlets. This requirement makes the establishment of sterile triploid *M.* x *giganteus* costly and labor intensive (Lewandowski et al, 2000). The estimated cost for mechanization of rhizome establishment was 350 Euro ha^{-1} ($128 $acre^{-1}$), and is expected to be reduced to 200 Euro ha^{-1} about US $74 $acre^{-1}$ in the future. Micropropagated plants, produced by *in vitro* culture, require much higher establishment costs of about 3000-6000 Euro (US$2730-5460) per hectare for typical densities of

one or two plants per square meter (Lewandowski et al., 2000). The cost of plant propagules and the need to propagate large numbers of plants are key factors constraining widespread planting of *M.* x *giganteus* (Atkinson, 2009). Micropropagation could allow the rapid multiplication of initial germplasm stocks selected in breeding programs. These materials would then undergo rhizome propagation to fullfill the requirement of large-scale establishment in the field. Micropropagation is also a viable approach for non-seasonal production, production of disease-free plants, germplasm conservation and facilitating their easy international exchange (Govil and Gupta, 1997). Increasing demand of *M.* x *giganteus* cultivation in various regions and countries requires improvement of current tissue culture micropropagation techniques to reduce propagule cost and increase availability.

In vitro propagation of *M.* x *giganteus* has been conducted by shoot regeneration from axillary nodes and apical meristems followed by *in vitro* tillering (Nielsen et al., 1993; Nielsen et al., 1995; Lewandowski, 1997), and by plantlet regeneration through somatic embryogenesis of callus induced from shoot apices, leaf sections and immature inflorescence tissues (Lewandowski, 1997; Holme and Petersen, 1996). Previous investigations have focused on optimization of tissue culture conditions to improve the induction rate of the embryogenic callus type, described as a compact opaque white callus, by addition of proline and different carbon sources, and the use of suspension culture methods in *M.* x *giganteus* (Holme et al., 1997; Petersen et al., 1999). Combination of auxin (2,4-dichlorophenoxyacetic acid, 2,4-D) and cytokinin (benzyladenine, BA) in callus induction medium increased the frequency of both embryogenic and shoot-forming callus induced from leaf sections and shoot apices compared with the medium containing auxin alone (Petersen, 1997). Relatively high regeneration rates of *M.* x *giganteus* callus was also achieved by the use of 2,4-D and BA when callus is induced from

immature inflorescence tissues (Lewandowski, 1997; Zhang, 2007). The medium condition previously employed to induce shoots from nodal segments of *M.* x *giganteus* (Nielsen et al., 1993) was tested for callus regeneration in our laboratory. Although it showed no superior effect for regeneration compared to the existing medium, relatively rapid regeneration trend was observed without data (Zhang, 2007).

The aim of this study was to elucidate the effect of callus types, ages and culture methods on regeneration efficiency under the existing *Miscanthus* tissue culture techniques. Different ages and types of callus maintained on either solidified medium or suspension cultures were tested to analyze the possible maintenance period of regenerable callus for long-term subculture. Improvement of regeneration efficiency with shortened tissue culture period was conducted through the culture of ideal callus types and ages chosen from our experiments. Regenerated plants were transplanted to field plots to evaluate winter survival, biomass productivity and somaclonal variation.

7.3. Materials and methods

7.3.1. Callus induction and maintenance culture

Immature inflorescence tissues, approximately 5-20 mm in length, were harvested from *M.* x *giganteus* plants grown in the greenhouse, and sterilized by immersion in 0.5% NaOCl solution for 3 min. Sterilized explants were cut into 5-7 mm sections and placed on callus induction medium (M1BA) consisting of MS basal salts and MS vitamins (Murashige & Skoog 1962) with 2,4-D (13.6 μM), BA (0.44 μM), 2.88 g L-proline, 30 g sucrose, and 750 mg $MgCl_2 \cdot 6H_2O$ as described by Petersen (1997). All media were supplemented with 2 g l^{-1} Phytagel™ (Sigma-Aldrich, St. Louis, MO), and adjusted to pH 5.5 prior to autoclaving. Fifteen explants were placed on each callus induction medium (M1BA) with fifteen replications.

Cultures were incubated in darkness at 27 ± 2 °C, and sub-cultured at 1-week intervals for the first two weeks and then at two-week intervals for four weeks. Six weeks after culture initiation, the total number of calli induced was recorded to calculate callus induction percentage. The different callus types were separated by their visual appearance and the number of each callus type was counted. Callus classification was as described by Lewandowski (1997), Holme and Petersen (1996) and Petersen (1997) for *M.* x *giganteus* callus evaluation. One callus unit was defined as 2 mm diameter calli. The percentage of each callus type was calculated as: callus type percentage = (number of each callus type / total number of callus pieces) x 100.

Two types of calli, compact and white callus (embryogenic-like callus) and a yellow or white compact callus with translucent and light-green shoot-like structures (shoot-forming callus), were maintained separately in solid or liquid callus maintenance medium for one year. Callus maintenance medium (M1) has the same composition as callus induction medium without BA. Ten pieces of calli (1-2 mm diameter) were transferred into each solid callus maintenance medium or 50 ml of liquid callus maintenance medium in 250 ml Erlenmeyer flasks, and the proliferated callus masses were subcultured by continuous selection for original callus type at two-week intervals. The flasks with liquid medium were shaken at 120 rpm at 27 ± 2 °C in darkness.

7.3.2. Plant regeneration

Effect of the callus types, ages and culture methods on the regeneration competence was studied with two different types of callus cultured on either solid or in liquid callus maintenance medium on two different regeneration media (MR1-1 and MR1-2) 1, 2, 4, 6, 8, 10 and 12 months after callus initiation. Each type and age of calli was transferred into the regeneration medium consisting of MS basal salts and MS vitamins (Murashige & Skoog 1962), 20 g sucrose and 750

mg $MgCl_2 \cdot 6H_2O$ with the addition of α-naphthaleneacetic acid (NAA) (1.3 μM) and BA (22 μM) for MR1-1 medium (Nielsen et al., 1993) and 2,4-D (4.5 μM) and BA (22 μM) for MR1-2 medium (Holme et al., 1997). Regeneration media was solidified with 3 g l^{-1} Phytagel™, and adjusted to pH 5.5 prior to autoclaving. Four Petri dishes with ten calli (3-4 mm diameter) each were plated out for each treatment, and subcultured at three-week intervals. Growth conditions were 16 hr of cool white fluorescent light (ca. 40μE) at 27 ± 2 °C. Shoots regenerating from callus were transferred into MS basal medium to induce roots, and rooted plantlets were later transferred to plastic pots (700 cm^3) containing a 1:1:1 (v/v/v) mixture of peat moss, vermiculite and perlite in the greenhouse. The number of plantlets regenerated in each treatment was recorded when they were transferred from regeneration to rooting medium during a four month period of incubation on regeneration medium. Multiple shoot clumps that could not be divided were considered as one plantlet. The regeneration frequency was calculated as the number of regenerated plantlets divided by total number of calli.

The incubation period required for callus differentiation into plantlets on regeneration medium was measured by counting the number of plantlets regenerated from shoot-forming calli after one month culture on MR1-1 and MR1-2. In this experiment, shoot-forming calli were induced from immature inflorescence tissues harvested from field grown *M. x giganteus* materials in July. Twenty calli per treatment were used for regeneration in five replications. To estimate the regeneration potential of calli remaining after removing the regenerated plantlets, the calli were subcultured on the same regeneration medium keeping the same replications for one additional month. The regeneration frequency of second set of calli was also calculated by same procedure.

7.3.3. Phenotypic evaluation and DNA content analysis of field-grown regenerated plants

Field plantings and growing of regenerated plants were conducted at the University of Illinois South Farm from 2006 to 2009. A total of fifty-four and twenty-five regenerated plants hardened in greenhouse for at least six months were planted in the spring of 2006 and 2007, respectively. Two morphological characteristics, plant height and stem diameter, were measured in fall 2009 at the mature flowering stage of the regenerated plants. Plant height was measured from the soil surface to the apex of the inflorescence, and stem diameter was measured as the width of stem at the 1 meter height of the stem from the soil surface with a slide caliper. Winter survival rate was calculated as the percentage of plants that survived the first winter season.

Five individual plants were selected from each of the 2006 and 2007 regenerated field plants based on their phenotypic variation in plant height, and DNA content of these plants compared with five plants randomly selected from rhizome propagated *M.* x *giganteus* plants. Approximately 500 mg of young and healthy leaf tissue of each regenerated plant were collected and ploidy levels determined by flow cytometry (Model LSRII, BD Biosciences at the University of Illinois-Keck Biotechnology Flow Cytometry Facility) as described by Rayburn et al. (1989, 2009) using sorghum nuclei as an internal standard.

7.3.4. Statistical analysis

Plant regeneration frequency of shoot-forming calli incubated on regeneration medium for one or two months, and phenotypic evaluation data obtained from field grown regenerated plants with replications were analyzed by the GLM procedure and LSD tests using SAS statistical analysis package (SAS version 8.0). DNA content collected from the field-grown regenerated plants were subjected to LSD test for significant differences between regenerated plants and rhizome propagated plants.

7.4. Results

7.4.1. Callus induction from immature inflorescence tissue

Immature inflorescence explants initiated callus about two weeks after culture on M1BA medium containing 13.6 μM 2,4-D and 0.44 μM BA at a frequency of 78 ± 7.8 % when measured six weeks after initiation. Small brown callus not distinguishable from the swollen explants were not counted. Three different types of callus were identified after six weeks of culture (Figure 2.1A-C). The dominant type of callus (41 ± 4 % of the total) was a yellow or white compact callus with light-green shoot-like structures (shoot-forming callus) (Figure 2.1A). The percentages of compact, white callus (embryogenic-like callus) (Figure 2.1B) and soft, friable callus (Figure 2.1C) were 22 ± 2.1 and 37 ± 3.7 %, respectively. We found that continuous subculture of shoot-forming calli on M1BA solid callus induction medium caused browning of most of calli. Maintenance of shoot-forming calli on solid medium containing only 2,4-D mostly produced yellowish and semi-soft callus type with a small portion of embryogenic-like callus type. Shoot-forming callus maintained in liquid medium also turned into the yellowish and semi-soft callus type, and most then formed roots on regeneration medium (Figure 2.1D).

7.4.2. Influence of callus type, age and culture maintenance method

The responses of the shoot-forming and embryogenic-like calli for plant regeneration are presented in Table 2.1. One month old shoot-forming calli showed the best regeneration performance compared with embryogenic-like calli. Dramatic reduction in regeneration potential with increasing age of shoot-forming callus culture was observed. After one month of subculture on solid callus maintenance medium, the estimated regeneration frequency of shoot-forming calli dropped to 0.3 and 0.1 plantlet per calli on MR1-1 and MR1-2 regeneration medium, respectively. No plants could be regenerated from the friable callus. Optimum concentration of

NAA and BA used in MR1-1 medium was obtained from our preliminary regeneration experiments conducted by treatment of eight combinations of NAA (1.3 and 5 μM) with BA (5, 10, 15 and 22 μM). Highest regeneration efficiency was found at the combination of 1.3 μM NAA and 22 μM BA (data not shown). Most of previous regeneration conditions employed with *M.* x *giganteus* were based on 22 μM BA (Holme and Petersen, 1996) or combination of 4.5 μM 2,4-D and 22 μM BA (MR1-2 medium) (Holme et al., 1997).

Regeneration of embryogenic-like callus type was less dependent on callus age compared with shoot-forming callus type. Continuous selection of the embryogenic-like callus type for two and four months of subcultures on solid medium increased the regeneration frequency, but after over five months of subculture no plant regeneration resulted regardless of callus types and regeneration medium. Suspension culture of embryogenic-like callus type in liquid medium did maintain regeneration competence for up to 12 months (Table 2.2). The highest plantlet regeneration was obtained from six month old embryogenic-like calli on MR1-2 medium. Regeneration frequency of suspension aggregates decreased after six months, but plantlets were still being obtained after one year of culture. Twenty percent of total regenerated plants obtained from eight month old suspension aggregates were albinos, whereas all other regenerated plants from different ages and types were normal green.

7.4.3. Rapid regeneration of shoot-forming callus

The effect of two auxin-like growth regulators, NAA or 2,4-D, on the regeneration efficiency and speed of plantlet regeneration from shoot-forming callus were further evaluated. Combination of NAA and BA in regeneration medium (MR1-1 medium) stimulated rapid shoot regeneration and growth from shoot-forming calli compared to 2,4-D and BA combination (MR1-2 medium) (Table 2.3). Regeneration frequency of shoot-forming calli on MR-1 medium

was significantly higher than the frequency obtained from MR1-2 medium during the first month of incubation, and total number of regenerated plants obtained after two months of incubation on regeneration medium was also significantly higher on MR1-1 medium. Most of regenerated plantlets (69 % of total regenerated plantlets) appeared within one month of incubation in MR1-1 medium, while only 30 % of total regenerated plantlets were produced in the same culture period on MR1-2 medium. Figure 2.1F and G taken after three weeks incubation of shoot-forming calli on regeneration medium showed the distinct differences on shoot regeneration and growing. Shoot-forming calli placed on MR1-2 medium formed many green spots indicating initiation of callus differentiation with few tiny multiple shoots on the surface of calli during three weeks (Figure 2.1G), but the growth of differentiated shoots were relatively slow compared to the regenerated shoots on MR1-1 medium (Figure 2.1F).

7.4.4. Phenotypic and cytogenetic variation of field grown regenerated plants

To determine if there were any phenotypic differences between field grown regenerated plants and rhizome propagated plants, two morphological characters, plant height and stem diameter were measured (Table 2.4). Plant height of regenerated plants planted in 2007 in high density plantings (0.3 m spacing) was significantly higher than rhizome propagated plants planted in 2004 and regenerated plants planted in 2006 in low density plantings (0.9 m spacing). Regenerated plants planted in low density planting were statistically superior in stem diameter but lower in plant height compared to the rhizome propagated plants and regenerated plants planted in high density planting. The average DNA content of regenerated plants selected by their short plant height phenotypes showed no significant differences with rhizome propagated plants and normal phenotype regenerated plants as a range from 7.19 ± 0.13 pg to 7.24 ± 0.05 pg

(Table 2.4). Winter survival rates of field planted regenerated plants in 2006 and 2007 were 78 % and 56 %, respectively.

7.5. Discussion

Our experimental data demonstrated that the types and ages of callus induced from immature inflorescence tissues are critical factors determining the efficiency of plantlet regeneration in *M.* x *giganteus*. Shoot-forming calli induced from immature inflorescence tissues showed best regeneration frequencies in our experiments. Plant regeneration was improved by using shoot-forming callus induced from leaf sections and shoot apices of *M.* x *giganteus* (Petersen, 1997). Combination of 2,4-D and BA in callus induction medium was effective to improve plant regeneration as well as induction frequency of shoot-forming callus in *M.* x *giganteus* (Petersen, 1997; Lewandowski, 1997). Maintenance of shoot-forming callus was not possible under our current culture conditions as was observed by Petersen (1997).

Loss of regeneration capability with increasing age of callus is a critical problem for callus maintenance and regeneration in *M.* x *giganteus,* as observed for other grass species (Cai et al., 1990; Brisibe et al., 1994). Holme et al (1997) established a suspension culture system to maintain embryogenic callus cultures in *M.* x *giganteus,* and high regeneration efficiency was obtained from the 18 months old suspension aggregates. Our experiments confirmed that the regeneration competence of embryogenic-like callus culture could be maintained for long-term culture periods (up to one year) by suspension culture. Fine selection and maintenance of a compact, white nodular callus type also seems to be an important factor affecting regeneration efficiency of embryogenic-like callus cultures. Regeneration frequency of embryogenic-like calli was increased after one or two months of subculture on solid callus maintenance medium. Although no regeneration was recorded from the relatively old callus materials in our first

experiment, the later experiments generated a few regenerated plants (data not shown). This kind of variation in plant regeneration could be due to callus types selected and maintained by continuous selection of embryogenic or proembryogenic callus type during subculture procedures. The embryogenic potential of wheat callus cultures could be maintained for twenty months by continuous selection of embryogenic clumps at subculture (Yang et al., 1991).

Regeneration response of embryogenic-like calli was relatively slow compared to shoot-forming calli which showed direct responses for regeneration under the same conditions. Although most of embryogenic-like calli initiated callus differentiation through the formation of green spots on the surface of calli, only few regenerated plantlets appeared within one month of incubation on MR1-2 regeneration medium, and then short multiple shoots developed from the differentiated callus later. Embryogenic-like calli continued to proliferate on MR1-2 regeneration medium.

Rapid and more synchronized regeneration from shoot-forming calli were distinct features that occurred on MR1-1 medium containing NAA and BA. Addition of NAA into the regeneration medium may stimulate shoot elongation of the calli already differentiated or undergoing cell differentiation like shoot-forming callus rather than undifferentiated calli. Poor effect of NAA and BA combination on regeneration of embryogenic-like callus type supports this possibility. Combination of NAA and BA was previously tested in our laboratory for *M.* x *giganteus* callus regeneration (Zhang, 2007). Although the regeneration experiments conducted without classifying callus types and ages on MR1-1 medium demonstrated comparable regeneration efficiency to MR1-2 medium (Zhang, 2007), the later repeated regeneration experiments displayed inconsistent regeneration performances (data not shown). The latter observation can be interpreted as the consequence of the regeneration from not uniform callus

types and ages. This result indicates that the selection of young shoot-forming callus morphotype is crucial for high efficiency of regeneration on MR1-1 medium.

Field grown regenerated plants showed normal phenotypic development with comparable plant height and stem diameter compared to rhizome propagated plants in our experiments. Planting of the mature rhizomes grown for 6-8 months in the greenhouse without rhizome cutting may accelerate the growth of the regenerated plants to reach the full standing height of *M.* x *giganteus*. Higher planting density did affect the development of field planted regenerated plants as these plants were significantly taller with lower stem diameters than in the low density planting (Table 2.4). Christian and Haase (2001) reported that high density planting of *Miscanthus* results in improved competition with weeds and the achievement of high yields more rapidly. It seems likely that the small phenotypic differences noted are due to microclimates and not somaclonal variation since the DNA contents of the regenerated plants and the rhizome propagated plants are not significantly different. It has been shown that regenerated plants produced through somatic embryogenesis were very competitive with rhizome propagated plants in terms of good morphological development and yield potential in *M.* x *giganteus* (Lewandowski, 1998; Clifton-Brown et al., 2007). Even higher shoot length and number of shoots per plant was observed in micropropagated plants over rhizome propagated plants in both the first and second ratoon by Lewandowski (1998). Micropropagated plants have proven to be more susceptible to winter losses than rhizome propagated materials (Lewandowski, 1998). Poor over-wintering of the rhizomes in the first year after planting was a problem in Northern Europe (Clifton-Brown and Lewandowski, 2000). Field tests of *M.* x *giganteus* in southern Ireland showed 60% and 53% of winter survival rate from rhizome propagated plants and micropropagated plants, respectively (Clifton-Brown et al., 2007). Our field experiments

showed 78 % and 56 % of winter survival rates from the regenerated plants transplanted in 2006 and 2007, respectively. Further study is required to elucidate the proper size of rhizomes and transplanting time for improvement of winter survival rate in field planted regenerated plants.

Our regeneration methods show a high frequency of plantlet regeneration with short tissue culture period that can be applied for large scale production of regenerated plants in *M.* x *giganteus*. Immature inflorescences are abundant materials which can be easily obtained from field-grown *M.* x *giganteus* during the summer or greenhouse grown plants throughout the year. Enough shoot-forming calli can be produced 6-8 weeks after culture of immature inflorescence tissues on M1BA medium, and most of the plantlet regeneration is achieved within two months on MR1-1 regeneration medium. Regenerated plantlets need to be hardened to form the proper size of rhizome during winter season in the greenhouse or winter nurseries, and then these propagules can be ready for large-scale field planting in the spring.

Table 7.1. Frequency of plant regeneration from shoot-forming and embryogenic-like calli on regeneration media MR1-1 and MR1-2.

Callus age[a] (month)	Regeneration frequency[b] of calli maintained on solid callus maintenance medium			
	MR1-1 medium (1.3 μM NAA and 22 μM BA)		MR1-2 medium (4.5 μM 2,4-D and 22 μM BA)	
	Shoot-forming calli	Embryogenic-like calli	Shoot-forming calli	Embryogenic-like calli
1	0.93	0.30	1.33	0.25
2	0.30	0.00	0.10	0.50
4	0.00	0.00	0.10	0.90
6	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00
12	0.00	0.00	0.00	0.00

[a]Callus age was time after placement on initiation medium.
[b]Regeneration frequency was estimated as the average number of regenerated plantlets per calli after four months incubation on regeneration medium with three weeks interval subculture (Forty calli were tested for each treatment).

Table 7.2. Effect of callus suspension culture on maintenance of regeneration competence for long-term subculture.

Callus age[a] (month)	Regeneration frequency[b] of calli maintained by suspension culture			
	MR1-1 medium (1.3 μM NAA and 22 μM BA)		MR1-2 medium (4.5 μM 2,4-D and 22 μM BA)	
	Shoot-forming calli	Embryogenic-like calli	Shoot-forming calli	Embryogenic-like calli
2	0.05	0.03	0.10	0.90
4	0.00	0.03	0.00	0.78
6	0.00	0.00	0.00	1.00
8	0.00	0.00	0.00	0.50
10	0.00	0.00	0.00	0.55
12	0.00	0.00	0.00	0.45

([a, b]: see table 7.1)

Table 7.3. Effect of two different regeneration media (MR1-1 and MR1-2) on rapid plant regeneration from six weeks old shoot-forming calli

Regeneration medium	Regeneration frequency		
	after one month incubation on regeneration medium[a, d]	after two months incubation on regeneration medium[b, d]	Total regeneration frequency[c, d]
MR1-1 (1.3 μM NAA and 22 μM BA)	0.84 ± 0.08a	0.35 ± 0.08a	1.19 ± 0.15a
MR1-2 (4.5 μM 2,4-D and 22 μM BA)	0.29 ± 0.05b	0.54 ± 0.07a	0.83 ± 0.08b

[a]Regeneration frequency was estimated as the number of regenerated plantlets divided by total number of calli after one month incubation on regeneration medium without subculture (Twenty calli were tested for each treatment with five replications).

[b]After taking out the regenerated plantlets from first month incubated calli materials, calli were subcultured and incubated on regeneration medium again for one more month, and then the number of regenerated plantlets was counted.

[c]Total number of regenerated plants obtained for two month of calli incubation on regeneration medium was used to estimate total regeneration frequency.

[d]Means within each column with different letters are significantly different at $P \leqq 0.05$, using Fisher's LSD

Table 7.4. Comparison of the phenotypic characters and DNA contents between plants derived from *in vitro* plant regeneration and rhizome propagation.

Plant origin	Number of lines	Plant height (meter) [a]	Stem width (cm) [a]	Winter survival rate (%)	DNA content (picogram/nuclei)
Regenerated plants planted in 2006 in low density planting[b]	18	3.17 ± 0.16b	1.03 ± 0.07a	78	7.19 ± 0.13a
Regenerated plants planted in 2007 in high density planting[b]	14	3.68 ± 0.13a	0.83 ± 0.05b	56	7.25 ± 0.01a
Rhizome propagated plants planted in 2004 in high density planting[b]	14	3.37 ± 0.10b	0.79 ± 0.03b	ND	7.24 ± 0.05a

[a]Means within each column with different letters are significantly different at $P \leqq 0.05$, using Fisher's LSD

[b]Spacing of low density planting and high density planting were 0.9 and 0.3 meter, respectively.

ND = Not Determined

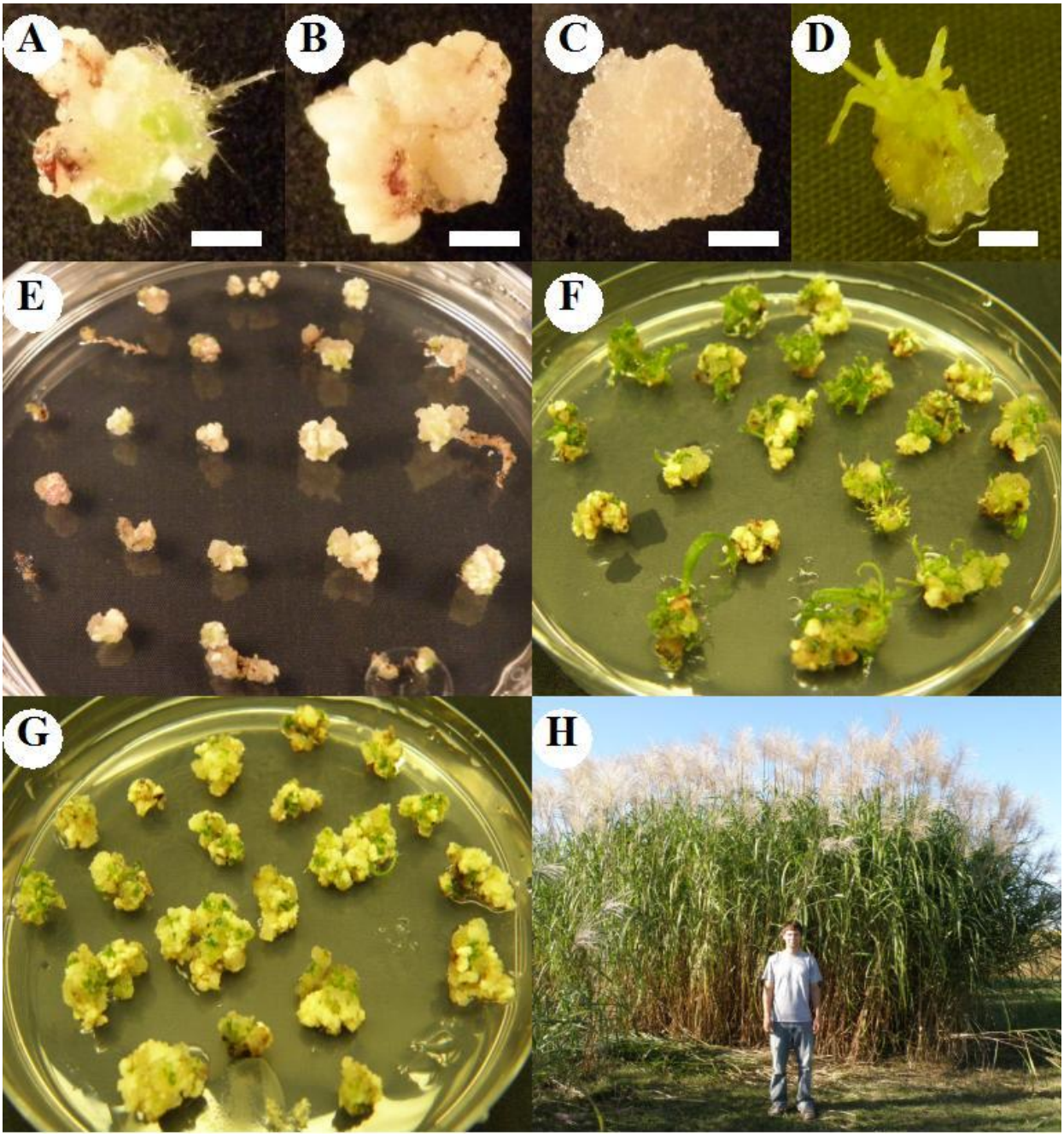


Figure 7.1. Plant regeneration from callus cultures of *Miscanthus* x *giganteus*. (A) shoot-forming callus induced from immature inflorescence tissue. (B) embryogenic-like callus. (C) soft, friable callus. (D) yellow, semi-soft, root-forming callus (bar = 1mm). (E) callus induction from immature inflorescence explants after three weeks incubation on M1BA callus induction medium. Callus differentiation into plantlets after three weeks incubation on MR1-1 medium (F) and MR1-2 medium (G). Plates are 10 cm in diameter. (H) Regenerated plants in the field in 2009. Person in the figure is 1.7 meter tall.

CHAPTER 8

Isolation of genes associated with lignin biosynthesis and flowering for vector construction and transformation of *Miscanthus* x *giganteus*

8.1. Abstract

Modification of lignin content and flowering time in bioenergy crops is a viable approach for improving biomass quality and productivity. Lignin is a key factor reducing hydrolysis efficiency since it forms covalent bonds with cellulose and hemicellulose polysaccharides in cell walls inhibiting enzyme access by cellulases during biomass degradation for hydrolysis and fermentation into ethanol or other liquid biofuels. In perennial C_4 *Miscanthus* species, late flowering extends the duration of vegetative growth and conversion of solar radiation into photosynthates, resulting in higher biomass yields. This study was conducted to provide basic information required to develop tools for the transformation of *Miscanthus* x *giganteus* to modify its cell wall composition and flowering time to enhance its potential as a dedicated biofuel crop

Seven lignin biosynthesis genes were isolated from *Miscanthus* x *giganteus* by PCR reactions using maize othologous sequences. These genes averaged 81% amino acid sequence similarity with the corresponding genes in *Sorghum bicolor*. Southern hybridization and nuclear DNA content analysis indicated that the lignin biosynthesis genes and a putative flowering gene (*ID1*) isolated from *M.* x *giganteus* exist in the genome of other *Miscanthus* species as multiple copies. Allotriploid *M.* x *giganteus* showed relatively high numbers of gene copies compared with diploid *M. sinensis* genotypes. Analysis of lignin content and histological staining of lignin deposition demonstrated higher levels of lignin in mature stem node tissues compared to young leaves and apical stem node tissues of *M.* x *giganteus*. This result suggests that degree of cell

wall lignification is associated with increasing tissue maturity in *Miscanthus* species. RNAi and antisense constructs harboring sequences of these genes were developed to generate *Miscanthus* transgenic plants with suppressed lignin biosynthesis and delayed flowering.

8.2. Introduction

The C_4 perennial rhizomatous grass species, *Miscanthus* x *giganteus* is a sterile allotriploid (3N=57) hybrid putatively generated from the natural cross between a diploid accession of *M. sinensis* (2N=38) and a tetraploid *M. sacchariflorus* (4N=76) (Greef et al,. 1997). With its high biomass productivity and relatively low input cost *M.* x *giganteus* has significant potential as a biofuel/bioenergy crop for temperate climates.

Lignin, cellulose, and hemicellulose are the major components of plant cell walls (van Wyk, 2001). Lignin forms covalent bonds with cellulose and hemicellulose polysaccharides in cell walls, and this structure inhibits enzyme access by cellulases during the biomass degradation process prior to fermentation. Lignin also inhibits microbial activity needed for the fermentation of cell-wall hydrolysates to ethanol. The low efficiency of conversion from biomass to fermentable sugar and the coproduction of toxic compounds in the presence of high lignin cell wall composition increase the expense of pretreatment and enzyme costs (Yuan et al., 2008). Plants with reduced lignin content are more easily digested into sugars and fermented into ethanol, enhancing crop value (McMillan et al., 1994). While lignin is essentially not fermentable, it is energy rich and chemically resembles soft coal (Greene et al., 2004). Alternatively, since lignin upon combustion generates more calories than cellulose, burning stems with higher concentrations of lignin should generate more heat and power. This suggests that genetic engineering to modify the cellulose/lignin ratios in *M. x giganteus* could result in transformed genotypes with either improved combustion or cellulosic ethanol productivity.

Modification of lignin content in biomass crops is considered a viable approach for improving biomass ethanol productivity and reducing costs for hydrolysis and fermentation. Although the functional study of the genes involved in monolignol biosynthetic pathway has been well investigated in various dicotyledonous plants such as *Arabidopsis*, *Populus*, tobacco and alfalfa (Franke et al., 2002; Lu et al., 2006; Blount et al., 2000), the same pathway has received only limited attention in monocotyledonous plants except for *Zea mays*. Monocots have a completely different vascular structure and distribution, compared to dicots and are believed to need an adequate lignin content in order to maintain stalk rigidity and normal development.

Although *M*. x *giganteus* is a sterile triploid (Greef and Deuter, 1993), it typically flowers in autumn sending up multiple showy inflorescences from each clone. The completion of flowering in *Miscanthus* species has been associated with the initiation of plant senescence (Mutoh et al., 1968) although low temperatures may also play a role. As with most grasses, flowering interferes with vegetative growth and consequently reduces biomass yield potential due to its termination of vegetative growth and competitive utilization of photosynthates for inflorescence development. The manipulation of genes that regulate the process of flowering induction in *Miscanthus* may be candidates for genetic engineering to develop transgenic genotypes with delayed flowering and enhanced biomass productivity. The *indeterminant 1* (*id1*) mutant in maize is an ideal candidate since a loss-of-function mutation at this locus was found to dramatically delay flowering and extend the vegetative growth period (Colasanti et al., 1998). Homozygous *id1/id1* mutants were much taller plants with more leaves, greater vegetative biomass and no or greatly delayed floral development. There is no available information about signaling pathways and key genes associated with *Miscanthus* flowering. Isolation and functional analysis of an orthologous *ID1* gene in *Miscanthus* will provide useful information to understand

the role of *ID1* and related flowering signaling pathways in *Miscanthus*. Artificial regulation of *Miscanthus* flowering time may also help to make new crosses between diverse *Miscanthus* species for trait improvement through the synchronization of flowering.

The aim of this study was to provide basic information required to improve biomass quality and productivity and develop tools for gene transformation in *M.* x *giganteus*. Seven genes associated with lignin biosynthesis and one putative flowering gene were isolated from *M.* x *giganteus* using primers based on the corresponding sorghum and maize gene sequences. The number of copies of the isolated genes and nuclear DNA content in *M.* x *giganteus* were analyzed to estimate gene redundancy and ploidy levels between *M.* x *giganteus* and purative parental *Miscanthus* species. *M. sinensis* and *M. sacchariflorus.* Lignin content and deposition were analyzed in different tissues (leaves and stems) of *M.* x *giganteus* at three developmental stages (seedling, vegetative stage and reproductive stage). Finally, RNAi and antisense constructs were prepared for future transformation of *Miscanthus* species to improve the biomass quality and productivity through the modification of lignin biosynthesis genes and flowering.

8.3. Materials and methods

8.3.1. Plant material

Tissue samples for DNA and RNA extraction were harvested from *M.* x *giganteus, M. sinensis* accessions 'Kascade', 'Goliath' and 'Amur Silvergrass' and the *M. sacchariflorus* accession, 'Robustus' plants grown in a greenhouse at the University of Illinois at Champaign-Urbana under a 25℃/15℃ and 14-h/10-h day/night temperature regime and with supplemental light. *M.* x *giganteus* was originally provided by the Chicago Botanic Garden. *M. sinensis* and *M. sacchariflorus* accessions were originally obtained from commercial horticultural nurseries.

Description on different tissue types and developmental stages of *M.* x *giganteus* plants used for lignin content measurement are shown in Table 8.1. Four plant subsamples were bulked in each of three replicates, frozen in liquid nitrogen, ground into powder, and stored at -20℃ until lignin content analysis.

8.3.2. Gene isolation from *M*. x *giganteus*

Total RNA was extracted from *M*. x *giganteus* leaf tissues using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The first strand of cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase with oligo-(dT) primer according to the instructions of the Reverse Transcription System (Promega, Madison, WI). Using known sorghum and maize gene sequence information, the primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3) to isolate seven *Miscanthus* othologous lignin biosynthesis genes (*C3H*, *C4H*, *F5H*, *4CL*, *COMT*, *CAD* and *CCR*) and one putative flowering gene (*ID1*) (Table 3.2). PCR amplification was performed using the GoTaq® PCR Core System (Promega, Madison, WI) following the protocol described by the manufacturer. Thirty five PCR cycles each comprising 1 min denaturation at 95℃, 1 min annealing at 60℃ and 2 min extension step at 72℃ were performed. The amplified PCR products were cloned with pGEM®-T Easy Vector System (Promega), and the clones were sequenced in the W. Carver Biotechnology Center, University of Illinois at Urbana-Champaign using the ABI 3730XL Capillary Sequencer (Applied Biosystems, Foster City, CA).

8.3.3. Southern hybridization

Southern blot analysis was conducted to estimate copy number of restriction fragments labeled with each gene isolated. Gene probes were designed to be small sized fragments (~500 bp) using the isolated *M.* x *giganteus* cDNA sequences to avoid restriction enzyme digestion

within the probe region. Plant genomic DNA was isolated from *M.* x *giganteus*, *M. sinensis* acessions 'Kascade', 'Goliath' and 'Amur Silvergrass' and *M. sacchariflorus*, 'Robustus' plants using the method described by McCouch et al. (1988). Twenty microgram of genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII or *Xba*I, separated by electrophoresis in 0.9% agrose gel, and then transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were hybridized with a ^{32}P-labeled probe, and washed at 60 ℃ for 10 min in 2 × SSC, 10 min in 1 × SSC, and 15 min in 0.5 × SSC with 0.1% (w/v) SDS.

8.3.4. DNA content analysis

Approximately 500 mg of young and healthy leaf tissue was collected from each *Miscanthus* accession, and nuclear DNA content and ploidy levels estimated by flow cytometry (Model LSRII, BD Biosciences at the University of Illinois-Keck Biotechnology Flow Cytometry Facility) as described by Rayburn et al. (1989, 2009) using sorghum nuclei as an internal standard.

8.3.5. Lignin analysis

Extraction and analysis of AcBr (acetyl bromide) lignin was conducted as described by Lygin et al. (2009). Alcohol insoluble residues were obtained from freeze-dried tissue samples after centrifugation and extraction with the solvent (70% methanol) to remove soluble components. The lignin content was calculated using the specific absorption coefficient (SAC) and following equation: percent of lignin content = (absorbance × 100)/SAC × sample concentration (g^{-1}).

8.3.6. Statistical analysis

Plant cell wall weight and AcBr lignin content obtained from different tissue types of *M.* x *giganteus* with replications were analyzed by the GLM procedure and LSD tests using the SAS statistical analysis package (SAS version 8.0).

8.3.7. Lignin histology

Lignin deposition in the middle and basal nodes of plants at 11 leaf stage, and the apical, middle and basal nodes at plant anthesis flowering were analyzed by histological staining of cell wall lignin as described by Aohara et al. (2009). Safranin O and methyl fast green were used to stain lignin red and other cell wall components blue. The middle node of each sample excised from *M*. x *giganteus* plants were fixed in a formalin-aceto-alcohol solution, dehydrated with a graded series of solutions with increasing ethanol composition, dried, and embedded in paraffin. Stem sections were cut at a thickness of 10 μm with a Leica RM2255 rotary microtome (LEICA, Bensheim, Germany), stained in 1% (w/v) safranin O (Sigma-Aldrich, St. Louis, MO) and 0.5% (w/v) methyl fast green (Sigma-Aldrich, St. Louis, MO) solution, and then visualized using Zeiss Stereolumar v12 microscope (Carl Zeiss, Thornwood, NY) in the Microscopy and Imaging Facility, University of Illinois at Urbana-Champaign.

8.3.8. Plant transformation vector construction for RNAi and antisense transformation

The lignin biosynthesis genes, *C3H*, *C4H*, *4CL*, *CAD*, *CCR* and *COMT* and the putative flowering gene, *ID1*, from *M.* x *giganteus*, were introduced into the RNAi and antisense constructs, pB7GWIWG2(II) and pB2WG7 (VIB, Leuven, Belgium), using the GATEWAY cloning system following the protocol described by the manufacturer (Invitrogen, Carlsbad, CA) (Figure 3.1). These constructs contained the herbicide resistance gene (*bar*) as a selection marker. Disarmed *Agrobacterium tumefaciens* strain EHA105 was used in a binary vector system. The

RNAi or antisense plasmid was introduced into *A. tumefaciens* strain EHA105 by the freeze-thaw method (An et al., 1988).

8.4. Results

8.4.1. Isolation of lignin biosynthesis genes and putative flowering gene

Eight *M.* x *giganteus* cDNAs encoding p-coumarate 3-hydroxylase (C3H), cinnamate 4-hydroxylase (C4H), ferulate 5-hydroxylase (F5H), 4-coumarate: coenzyme A ligase (4CL), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl-CoA reductase (CCR) involved in lignin biosynthesis, and indeterminante 1 (ID1) related with flowering were isolated by PCR reactions using primers based on maize or sorghum othologous gene sequences. The amino acid sequences deduced from the isolated *M.* x *giganteus* cDNA sequences corresponded to the target sorghum proteins overall by 81%, and averaged 81% amino acid sequence similarity with the corresponding sorghum sequences (Table 8.3). The three cytochrome *P450* genes, *C3H* (*CYP98*), *C4H* (*CYP73*), and *F5H* (CYP84), exhibited relatively higher amino acid sequence similarity with the corresponding sorghum and maize sequences, indicating highly conserved *P450* gene sequences across different plant species. Relatively low sequence similarity of *COMT* gene between species was consistent with the previous observations by Xu et al. (2009).

8.4.2. Variation in the gene copy number in *Miscanthus* species

Southern hybridization revealed that the lignin biosynthesis genes and the putative flowering gene isolated from *M.* x *giganteus* exist in the genome of other *Miscanthus* species as multiple copies (Table 8.4). The number of gene copies was counted to compare the level of gene duplication between *Miscanthus* species having different ploidy levels (Table 8.4 and Table 8.5). Allotriploid *M.* x *giganteus* showed relatively high numbers of putative lignin and

flowering gene copies compared with diploid *M. sinensis* genotypes, suggesting potential combination of divergent genomes in *M*. x *giganteus*. The number of gene copies labeled by *4CL* and *CCR* gene probes were relatively lower than the reference information obtained from sorghum and rice genome (Table 8.3). This may be due to the limited sequence homology of our gene probe with the entire gene family. The *4CL* and *CCR* were multiple family genes in the maize genome (Guillaumie et al., 2006). Although our experiment confirmed the existence of the isolated genes in *Miscanthus* genome, proof of copy number and gene duplication await further development of lignin and flowering gene sequences and marker development. It also needs to be considered that gene expression may be influenced by the condition of polyploidy (Adams and Wendel, 2005; Chen and Ni, 2006). Recent studies have demonstrated that transgene-induced post-transcriptional gene silencing is not affected by ploidy level (Pignatta et al., 2008). This result suggests that the use of transgene-driven post-transcriptional gene silencing could be a viable approach to modify gene expression in polyploid species.

DNA content data was useful to estimate the putative hybridization event that occurred to produce the allotriploid *M.* x *giganteus* (Table 8.5). Hypothetically, crossing diploid *M. sinensis* (2N) with tetraploid *M. sacchariflorus* (4N) would generate an estimated DNA content of 7.54 pg, close to that observed in *M.* x *giganteus* (7.35 pg).

8.4.3. Variation in lignin content and deposition in different tissues and developmental stages of *M.* x *giganteus*

Young seedling and leaf tissues displayed the lowest level of AcBr lignin content, whereas mature stem tissues showed relatively high levels of lignin (Figure 8.2). Increasing lignin content in middle nodes leaf and stem tissues with developmental from the vegetative to reproductive phase suggests that lignification is related to plant age. Histological staining of cell

wall components also indicates cell wall greater lignification with increasing maturity of the plant (Figure 8.3). Increasing distribution and size of vascular bundles with a thicker epidermal layer were common features observed in basal stem node sections in plants at the 11-leaf stage and middle and basal stem nodes at flowering stage of *M.* x *giganteus* (Figure 8.3).

8.4.4. Preparation of plant transformation vectors for gene functional studies in *M.* x *giganteus*

The application of post-transcriptional gene silencing using RNAi or antisense gene constructs was employed to generate differences in PTGS efficiency in potential transgenic *Miscanthus*. Phenotypic variation of transgenic plants generated by RNAi or antisense approaches may help to produce elite transgenic plants having desirable phenotypes with improved biomass productivity in *Miscanthus.*

The lignin biosynthesis genes, *C4H*, *C3H*, *4CL*, *CAD*, *CCR* and *COMT*, and putative flowering gene, *ID1*, were introduced into RNAi and antisense constructs for application of transgene-based post-transcriptional gene silencing in *M.* x *giganteus* callus tissue (Figure 8.1 and Table 8.6). Transformation with genes involved in lignin biosynthesis and flowering has the potential to yield transgenic germplasm with enhanced biofuel productivity and increased biomass accumulation in *Miscanthus* species.

8.5. Discussion

The generation of new variation *in M.* x *giganteus* may best be achieved by genetic transformation due to sterility of this allotriploid. Conventional breeding of *Miscanthus* has serious limitations due to the difficulty in generating viable seed from sexual hybridization between the *Miscanthus* parental species and sterility of the triploid *M.* x *giganteus*.

Development and deployment of transgenic *M.* x *giganteus* genotypes would have a reduced invasive risk since the plant has sterile pollen and will not set seed (Hernández et al., 2001).

The low efficiency of conversion lignocellulosic biomass to fermentable sugar and the coproduction of toxic compounds in the presence of high lignin content in feedstocks increase the expense of pretreatment and enzyme costs. The modification of lignin content is considered a viable approach for this objective, since plants with reduced lignin content are more easily digested into sugars, which is then fermented into ethanol (McMillan et al., 1994). The *brown midrib* mutations (*bm1, bm2, bm3,* and *bm4*) in maize are associated with significant alterations in lignin composition and content in stover (Petersen et al., 2005). The *bm1* mutation reduces CAD activity (Haplin et al., 1998) while *bm3* results in reduced COMT activity (Vignols et al., 1995). Reduction in lignin content of maize has recently been accomplished by down-regulation of *COMT* using antisense constructs, with transgenic plants showing very similar characteristics to the maize *bm3* mutant phenotype (Piquemal et al., 2002). Down-regulation of *COMT* to low activity levels also reduces lignin content in alfalfa, maize, and poplar by 30%, 30%, and 17%, respectively (Guo et al., 2001; Chabbert et al., 1994; Jouanin et al., 2000). These results emphasize the importance of genetic modification and functional characterization of lignin biosynthesis genes in order to develop commercially valuable genotypes as biofuel crop resources.

Table 8.1. Description on different tissue types sampled at three developmental stages of *M*. x *giganteus* growth. Tissue samples were harvested from plants grown in a greenhouse.

Developmental stage	Sample number	Tissue type	Sample description
6-leaf stage	1	Seedlings	Whole seedlings
11-leaf stage	2	Apical leaves	9th-12th leaves from the stem base
	3	Middle leaves	5th-8th leaves from the stem base
	4	Middle stems	3rd-4th internodes from the stem base
	5	Basal stems	1st-2nd internodes from the stem base
Flowering stage	6	Apical leaves	9th-12th leaves from the stem base
	7	Apical stems	5th-6th internodes from the stem base (below inflorescence)
	8	Middle leaves	5th-8th leaves from the stem base
	9	Middle stems	3rd-4th internodes from the stem base
	10	Basal stems	1st-2nd internodes from the stem base

Table 8.2. List of primers used for gene isolation in *M.* x *giganteus*.

Target gene	Reference gene (Accession number)	Forward Primer (5'-3')	Reverse Primer (5'-3')
Lignin biosynthesis			
MgC3H[x]	*SbC3H*[y] (XM_002458602) and *ZmC3H*[z] (BT087676)	TGGTACACAAACTCGCAACAG	ACCGTGAAGATCATGGAGGA
MgC4H	*SbC4H* (AY034143) and *ZmC4H* (NM_001155686)	5' portion GTACTCGAAGCTCTGCGACA	GTTCGGCGACATCTTCCTC
		Core portion ATCAACGAGGACAACGTGCT	ACTGAACTGGCCTCCCTTCT
		3' portion AGGCCATCGTCAAGGAGAC	CTTCTCCGTGGTGTCGATCT
MgF5H	*SbF5H* (XM_002464338) and *ZmF5H* (EU957645)	5' portion CACTACGGGCCCTTCTGG	GTGCATCATCTCCGCCATC
		3' portion CAACATCAAGGCCATCATCA	AGACGTCGCTCATGTCCAG
Mg4CL	*Sb4CL* (XM_002451602) and *Zm4CL* (NM_001111788)	5' portion GACATCGAGATCGACAGCAG	CACCGCGTTCAGCGAGTAGA
		3' portion GCCGCTGTTCCACATCTACT	CGACCTCCTTTGCAACAAAC
MgCOMT	*SbCOMT* (AY217766) and *ZmCOMT* (M73235)	5' portion GTGCATGTACGCGATGCAG	CAGTCGTGGAGGATCCACTT
		3' portion ACGCTGAAGAACGCCATC	GCGTTGGCGTAGATGTAGGT
MgCAD	*SbCAD* (AB288109) and *ZmCAD* (NM_001112184)	5' portion GTCCGAGAGGAAGGTGGTC	CGGGATCTTCACCACAAACT
		Core portion AGGCCAACGTTGAGCAGTA	ATGCTGCCGATGAAGCTC
		3' portion ATCAGCTCGTCGTCCAAGAA	CTCGTTCACGTACCCCATCT
MgCCR	*SbCCR* (XM_002437926) and *ZmCCR* (X98083)	5' portion CACGAGGGGGATACACTGTC	CCGTCCAGGTACTTGAGCA
		3' portion AACAGCAGAGGCGACGAC	CTTTCTCCTGGAGGCTGGT
Flowering			
MgID1	*SbID1* (XM_002467169) and *ZmID1* (NM_001111439)	AGATCTGCAACAAGGGGTTC	CGAGGAAGTCCCTAGTGAGG

[x, y, and z] *Mg*, *Sb* and *Zm* indicate *Miscanthus* x *giganteus*, *Sorghum bicolor* and *Zea mays*, respectively.

Table 8.3. Comparison of the isolated *M*. x *giganteus* gene sequences with corresponding sorghum gene sequences.

Designation	Identity of the deduced amino acid sequences (%) [x]	Amino acid sequence similarity (%) [x y]
	Sb[z] / Mxg	Sb / Mxg
Lignin biosynthesis / size of the isolated Mxg cDNA (bp)		
C3H / 1233	100 / 80	100 / 93
C4H / 1399	100 / 93	100 / 84
F5H / 1398	100 / 88	100 / 95
4CL / 1497	100 / 89	100 / 90
COMT / 576	100 / 53	100 / 49
CAD / 1009	100 / 92	100 / 90
CCR / 821	100 / 79	100 / 62
Flowering		
ID1 / 992	100 / 70	100 / 83

[x]Sequence identity indicates the percentage of the isolated sequences corresponding to the sorghum protein.
[y]Sequence similarity is the percentage of the positively aligned amino acid sequences within the overlapped region between *M*. x *giganteus* and sorghum protein. Sequence identity and similarity were based on ClustalW results (Thompson et al., 1994).
[z]Sb and Mxg indicate *Sorghum bicolor* and *M.* x *giganteus*, respectively.

Table 8.4. The estimated monolignol biosynthetic gene copy number in sorghum, *M.* x *giganteus*, *M. sinensis* acessions 'Kascade', 'Goliath' and 'Amur Silvergrass', and *M. sacchariflorus*, 'Robustus'.

Plant species and genotypes	Estimated number of gene copies [x]							
	C3H	*C4H*	*F5H*	*4CL*	*COMT*	*CAD*	*CCR*	*ID1*
Sorghum	2	4	1	2	3	2	3	3
M. sinensis acessions 'Kascade'	6	6	3	2	3	4	3	2
M. sinensis acessions 'Goliath'	2	2	2	1	4	4	3	3
Putative *M. sinensis* 'Amur Silvergrass'	6	5	1	3	3	5	3	1
M. x *giganteus*	5	9	4	5	4	5	4	4
M. sacchariflorus, 'Robustus'	5	10	2	3	3	3	5	3
Plant species and genotypes	Number of genes in sorghum and rice[y]							
	C3H	*C4H*	*F5H*	*4CL*	*COMT*	*CAD*	*CCR*	*ID1*
Sorghum	2	4	3	12	9	5	12	ND [z]
Rice	2	4	3	12	9	5	12	ND

[x]Minimal number of hybridizing bands generated by multiple restriction enzyme digestions was counted as gene copy numbers to avoid over estimating copy numbers caused by high gene heterozygosity among *Miscnthus* species.

[y]The numbers of genes in sorghum and rice genome were based on the sorghum and rice genome annotation data and blast results reported by Xu et al. (2009).

[z]Not determined.

Table 8.5. Comparison of mean nuclear DNA content among different *Miscanthus* accessions.

Species	Accession	Nuclear DNA content (pg)
M. sinensis	Kascade	5.72 ± 0.10 c
	Goliath	8.11 ± 0.10 a
	Amur Silvergrass	5.44 ± 0.19 d
M. x *giganteus*	UIUC	7.35 ± 0.06 b
M. sacchariflorus	Robustus	4.68 ± 0.10 e

Means with a different letter are significantly different at P ≦0.05, using Fisher's LSD analysis.

Table 8.6. List of plant transformation vectors prepared for suppression of lignin biosynthesis gene and putative flowering gene in *M.* x *giganteus*.

Vector name	Target gene	Size of gene sequence (bp)	Description
pMGRi-C3H	*C3H*	500	RNAi for suppression of *C3H* gene
pMGAS-C3H	*C3H*	865	Antisense for suppression of *C3H* gene
pMGRi-C4H3	*C4H3*	500	RNAi for suppression of *C4H3* gene
pMGRi-4CL	*4CL*	500	RNAi for suppression of 4*CL* gene
pMGRi-COMT	*COMT*	500	RNAi for suppression of *COMT* gene
pMGAS-COMT	*COMT*	670	Antisense for suppression of *COMT* gene
pMGRi-CAD	*CAD*	500	RNAi for suppression of *CAD* gene
pMGRi-CCR	*CCR*	500	RNAi for suppression of *CCR* gene
pMGRi-ID1	*ID1*	500	RNAi for suppression of *ID1* gene
pMGAS-ID1	*ID1*	1100	Antisense for suppression of *ID1* gene

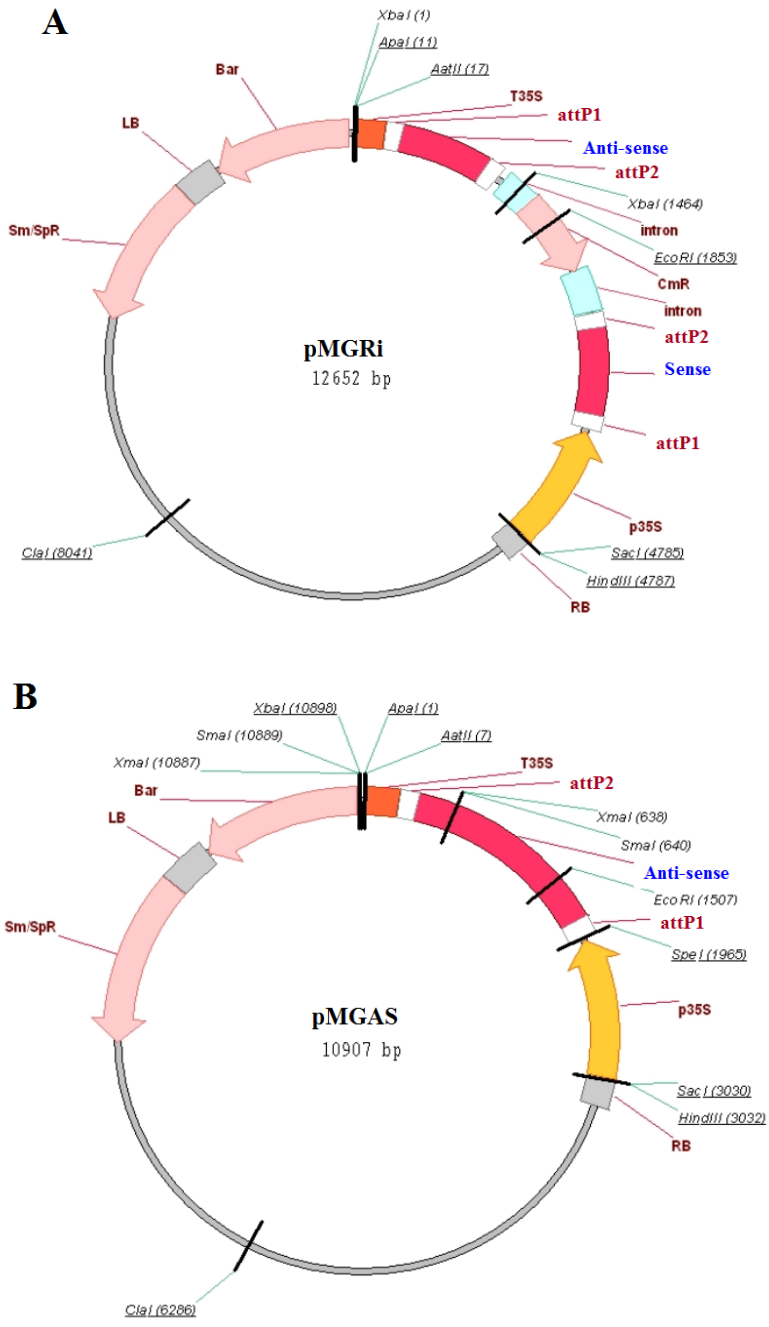


Figure 8.1. Completed RNAi (A) and antisense (B) constructs for *Agrobacterium*-mediated transformation in *M.* x *giganteus*. [Figure source: http://gateway.psb.ugent.be]

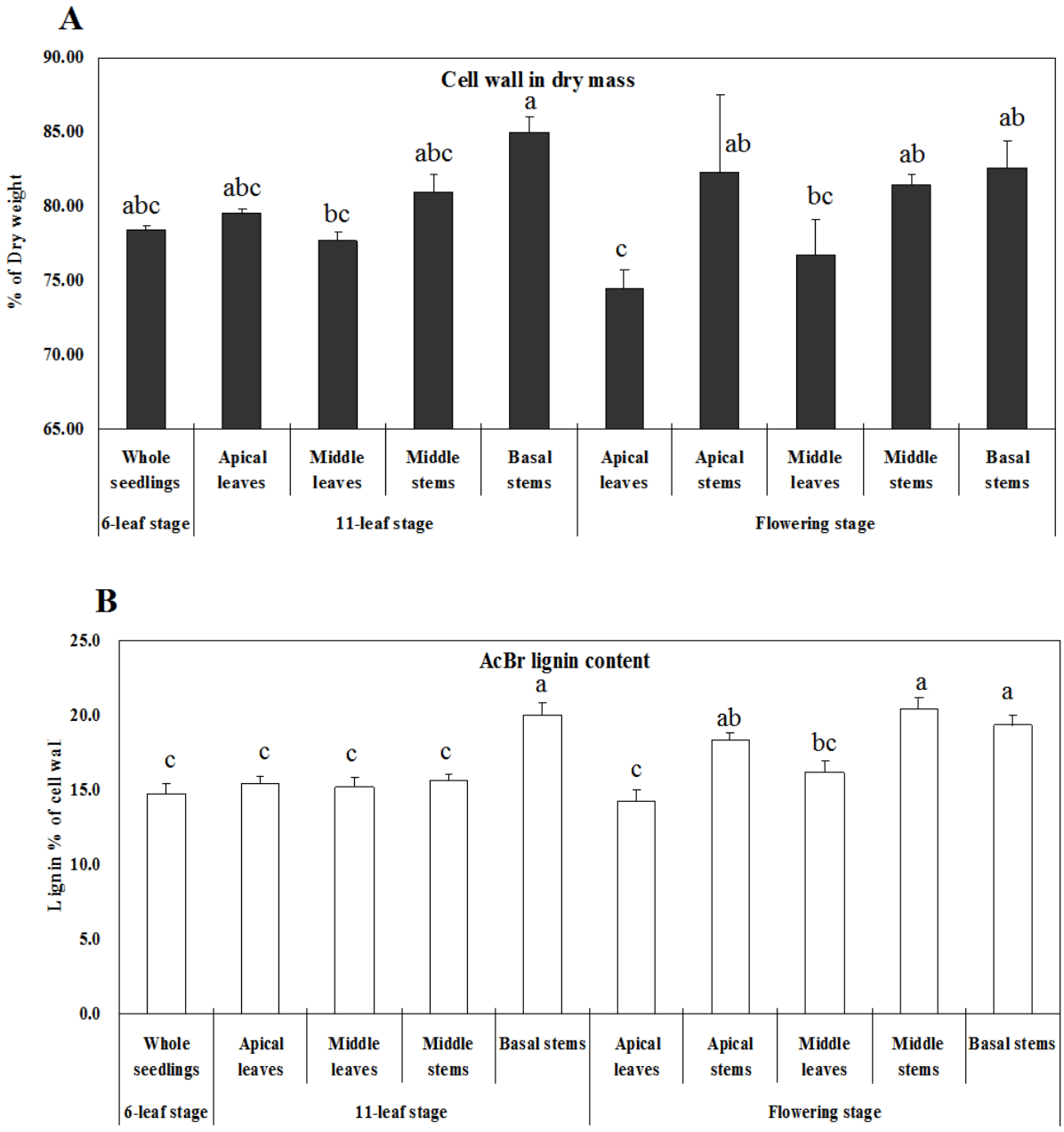
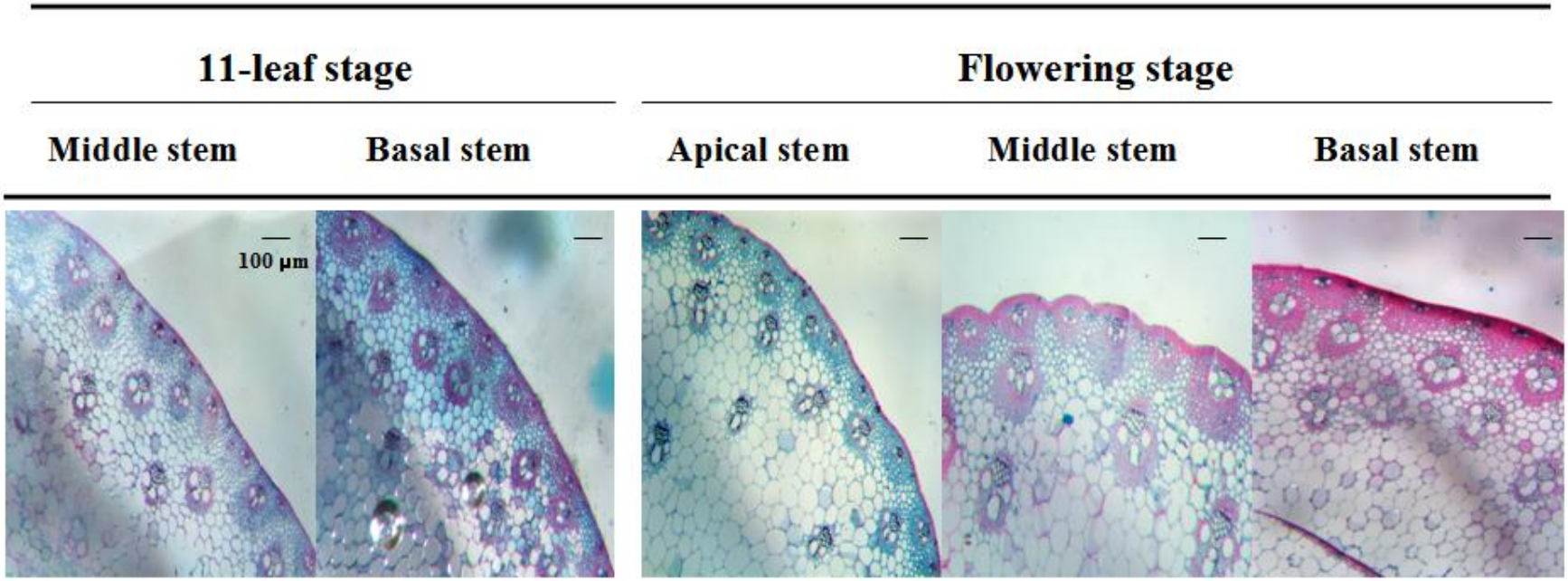


Figure 8.2. Variation in cell wall dry weight and AcBr lignin content in different tissues at three developmental stages (seedling, vegetative and flowering stages) of *M.* x *giganteus.* Means with a different letter are significantly different at P ≦0.05, using Fisher's LSD analysis.

Figure 8.3. Histological staining of cell wall lignin stem at two developmental stages (vegetative and flowering stage) of *M.* x *giganteus* growth. Images shown are representative of at least four independent experiments. Bars = 100 μm.



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